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PART I

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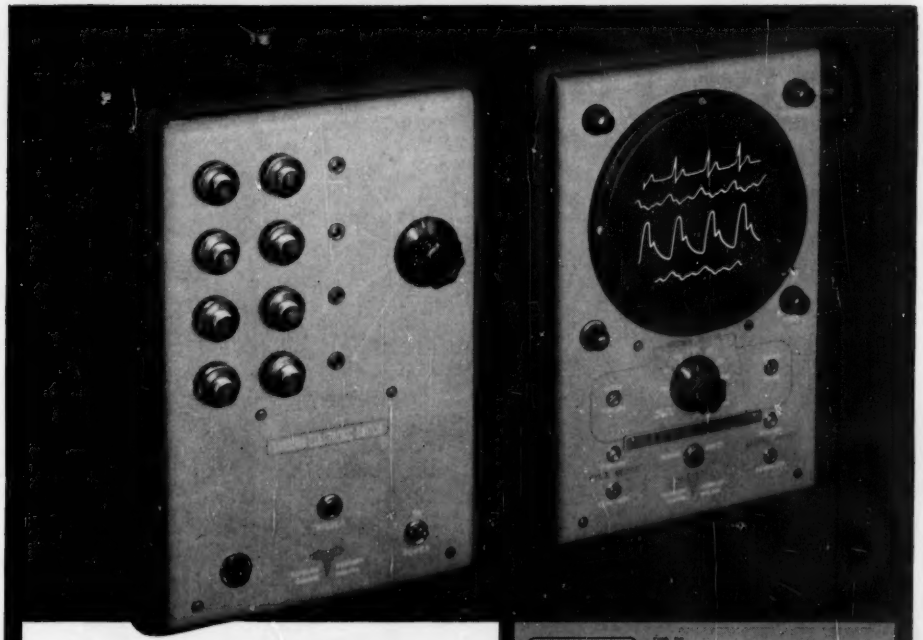

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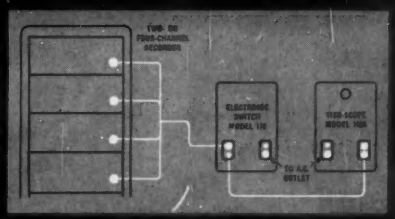

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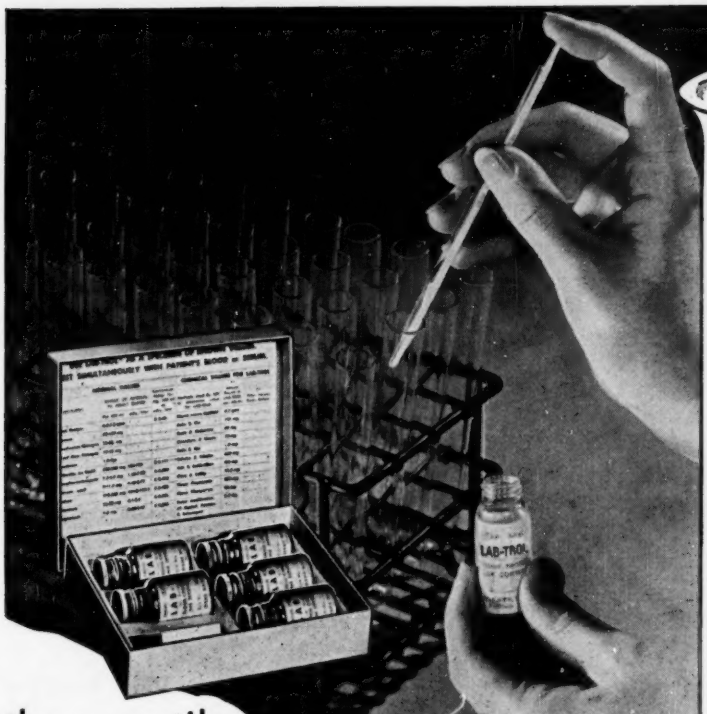

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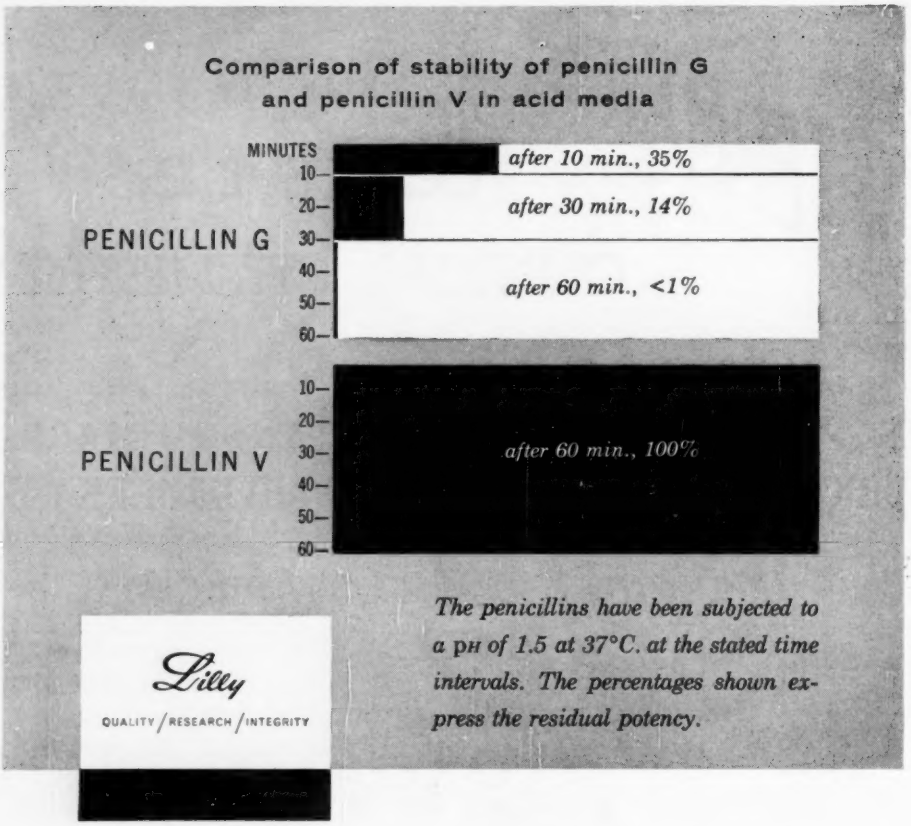
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A STANDARDIZED BREATH HOLDING TECHNIQUE FOR THE CLINICAL MEASUREMENT OF THE DIFFUSING CAPACITY OF THE LUNG FOR CARBON MONOXIDE [1]

By C. M. OGILVIE,[2] R. E. FORSTER,[3] W. S. BLAKEMORE,[4] AND J. W. MORTON [5]

(From the Departments of Physiology and Pharmacology, and Anesthesiology, the Graduate School of Medicine, and the Harrison Department of Research Surgery, School of Medicine, University of Pennsylvania, Philadelphia, Penna.)

(Submitted for publication April 16, 1956; accepted August 13, 1956)

Previous publications from this laboratory have described (1, 2) a modification of the Krogh breath nolding technique for measuring the pulmonary diffusing capacity for carbon monoxide (D_L).[6] This test can be performed quickly and simply and does not require arterial blood analyses. It has recently (2) been shown to provide an index of pulmonary diffusing capacity similar to that given by the $D_{L_{O_2}}$ method of Lilienthal, Riley, Proemmel, and Franke (3) and the "steady state" method of Filley, MacIntosh, and Wright (4). The purpose of the present report is to enumerate some of the factors that affect D_L, to describe a standardized technique for its measurement, and to present normal values of D_L as well as values in patients with various chest diseases.

[1] Supported by a grant from the Life Insurance Medical Research Fund.

[2] Travelling Fellow of the Postgraduate Medical Federation, University of London. Present address: The London Hospital, London.

[3] Lowell M. Palmer Senior Fellow.

[4] Scholar in Cancer Research of the American Cancer Society.

[5] Present address: Division of Tuberculosis Control, Willow Chest Center, 2647 Willow Street, Vancouver 9, B. C.

[6] Since the combination of CO with intracellular hemoglobin occurs at a rate that offers an appreciable, and under some conditions the major, part of the resistance to the uptake of CO in the lungs (5, 6) it is desirable to distinguish between the "true" diffusing capacity, or that of the pulmonary capillary membrane alone (D_m), and the apparent diffusing capacity of the whole lung (D_L). These are related by the equation $1/D_L = 1/D_m + 1/\theta V_c$ where θ is the rate of combination of CO with intracorpuscular hemoglobin in ml. per min. per mm. Hg CO tension per ml. blood, and V_c is the volume of blood in the pulmonary capillaries at any instant. θ decreases as O_2 tension increases (7), causing D_L to decrease, since D_m and V_c are presumably relatively independent of alveolar O_2 tension (8). Normally a further subscript of CO or O_2 would be used to indicate the gas to which the measurement applies. However, in this article, which is mainly concerned with CO, the subscript is omitted and can be assumed to be "CO" unless otherwise stated.

METHODS

The technique for the measurement of D_L, which has been reported before (1, 2), consists essentially of having the subject make a maximal inspiration of a gas mixture containing 10 per cent helium (He), 0.3 per cent CO and approximately 21 per cent O_2 in N_2 from the level of his residual volume, hold it for a measured time, and then rapidly expire. All of this expiration except the first liter is collected in a bag by the operator and analyzed as alveolar gas. The CO concentration which was present in this sample before any CO had been absorbed in the lungs is calculated from the dilution of the inspired He according to the equation,

$$\text{Initial CO concentration in the expired alveolar sample} = \frac{\text{He concentration in the expired alveolar sample}}{\text{Inspired He concentration}} \times \text{Inspired CO concentration} \quad (1)$$

Knowing the change in CO concentration in the alveolar sample during the period of breath holding, D_L can be calculated from Krogh's equation (9).

$$D_L \left[\frac{\text{In ml. CO STPD}}{\text{Min.} \times \text{mm. Hg CO tension}}\right] = \frac{\text{Alveolar volume (STPD) } 60}{\text{Time in seconds} \times (\text{barometric pressure} - 47)} \times \text{Natural logarithm} \left[\frac{\text{Initial CO concentration in the expired alveolar sample}}{\text{Final CO concentration in the expired alveolar sample}}\right] \quad (2)$$

The apparatus used for the test is illustrated in Figure 1. It differs from that reported previously (1, 2) mainly in its greater simplicity; in these previous communications the expired alveolar He and/or CO concentrations were measured at the mouth by recording analytical instruments. The circuit is closed, permitting inspiration from the bag of the Donald-Christie apparatus and expiration into the space around the bag, a spirometer recording the change in respiratory volumes. Tap (A) is used by the operator for collecting the expired alveolar sample. Since the gas mixture is inspired from the level

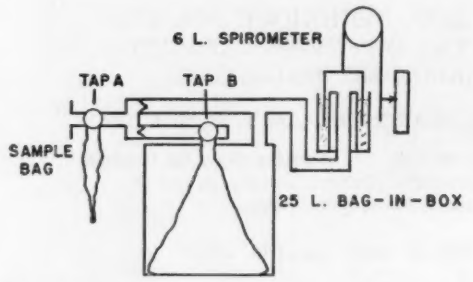

FIG. 1. DIAGRAM OF THE APPARATUS FOR MEASURING D_L IN PATIENTS

of the residual volume, the alveolar volume equals the residual volume,[7] which was measured by a modification of the He closed-circuit technique (10) or by the nitrogen wash out method (11), plus the inspired volume, which was measured directly from the spirometer tracing. The duration of breath holding was timed from the start of inspiration to the start of the collection of the alveolar sample as measured on the spirometer tracing (paper speed 0.44 cm. per second; precision $\pm$ 0.1 seconds). A breath holding period of ten seconds was chosen because this is long enough to produce an accurately measurable fall in alveolar CO concentration and yet is well within the breath holding limits of most patients.

As previously described (1), the alveolar gas samples were analyzed for CO in an infra-red analyzer,[8] after passing over calcium chloride. Correction for CO_2 interference should be made where necessary but the CO_2 sensitivity of the analyzer used in these studies was found to be negligible, 5 per cent CO_2 producing a deflection equivalent to less than 0.0005 per cent CO. Readings of percentage CO were precise to within $\pm$ 0.001 per cent.

The alveolar gas samples were analyzed for He in a catharometer.[9] Since the catharometer actually measures the total thermal conductivity of the gas mixture, changes in the concentrations of O_2, N_2 (argon included) and CO_2 will affect the measurement, in addition to changes in the concentration of He. It was sufficiently accurate to make standard corrections for the presence of O_2 and N_2, but for CO_2 it was necessary to measure the relative thermal conductivity of the gas sample before and after absorption of CO_2. The analyses of He were precise to within $\pm$ 0.05 per cent. About 300 ml. of gas were required for the analysis of CO and He.

A test and a duplicate could be completed within 5 to 10 minutes, but an additional hour was required for the gas analyses and calculations. This does not include the time needed to estimate the residual volume. In an effort to obviate this latter measurement we attempted to calculate the residual volume from the inspired volume and the dilution of He in the expired alveolar sample, but found that uneven distribution of inspired gas rendered the resulting value so unreliable as to be useless in all but extreme circumstances.

A motor-driven treadmill set at a gradient of 8 per cent was used for all exercise studies at speeds up to 4.5 miles per hour. For the measurement of minute volume and O_2 consumption during exercise breathing air, the expired gas was collected in a 300-liter rubber bag through a low resistance valve. The volume of this gas was measured in a spirometer and the N_2, O_2 and CO_2 concentrations analyzed in a Scholander apparatus (12). O_2 consumption was calculated from these data.

Determinations of lung volumes, including residual volume, maximum breathing capacity, maximal expiratory and inspiratory velocities between 200 and 1200 ml. by the method of Danzig and Comroe (13) and uniformity of distribution of inspired gas by the single breath method of Comroe and Fowler (14) were performed on each patient in the pulmonary function laboratory. Subject 22 and Patients 2, 8, 11 and 24 were studied by bronchospirometry and D_L measured in each lung.

SUBJECTS

Twenty-eight normal subjects (Table I), 16 males and 12 females, ranging in age from 8 to 72 years and in surface area from 0.98 to 2.12 square meters were studied. Most of these subjects were either physicians or laboratory personnel, who had no significant history of pulmonary or cardiac disease, were able to exercise without discomfort, and reported no pulmonary abnormalities by x-ray or physical examination. The remaining subjects had no significant pulmonary disease by history, physical, or x-ray examination.

RESULTS AND DISCUSSION

A. Some Factors Influencing D_L and Their Control

None of the subjects or patients experienced any difficulty in performing the test and all were able to hold their breath for the ten-second period. However, a number of factors either affected, or might be expected to affect, D_L as measured by the present technique. These factors, their importance in obtaining reproducible estimates of D_L, and the measures that were taken to control them are discussed below.

Measurement and duration of the breath holding period

It can be seen from Equation 2, that any error in the measurement of the time of breath holding

[7] More properly, the respiratory dead space should be subtracted from the residual volume. We have neglected this correction, which leads to an overestimate of D_L by about 3 per cent in normals.

[8] Liston-Becker, Stamford, Conn.

[9] Cambridge Instrument Company, Cambridge, England.

TABLE I

D_L in healthy subjects—relationship to age, sex and body size

Group	Subject	Age *years*	Height *inches*	Weight *pounds*	Surface area M^2	No. of observations of D_L	Mean D_L* in $\frac{ml.}{min. \times mm.\ Hg}$
Young Normal (8–35 years)							
Male	1	33	72.0	164.0	1.96	11	26.5
	2	34	66.0	156.0	1.81	2	24.9
	3	32	67.5	134.0	1.72	21	32.8
	4	35	74.0	165.0	2.01	7	34.1
	5	35	67.0	175.0	1.91	2	29.0
	6	32	69.5	157.0	1.87	5	34.0
	7	8	53.0	61.0	1.02	2	11.0
	8	10	55.0	72.0	1.12	2	16.5
	9	20	73.0	175.0	2.04	9	37.5
	10	32	73.0	170.0	2.00	2	34.8
	11	10	51.0	59.0	0.98	2	17.3
	Mean	25.6	65.6	135.3	1.68	6.0	27.1
Female	12	23	62.5	102.0	1.45	8	19.1
	13	27	67.5	215.0	2.12	2	34.8
	14	20	64.5	116.0	1.55	5	29.1
	15	22	65.0	140.0	1.70	2	22.2
	16	21	70.0	124.0	1.70	2	18.8
	17	26	65.5	109.0	1.49	2	16.4
	18	30	70.5	140.0	1.80	2	27.1
	19	26	65.0	122.0	1.60	3	23.8
	20	18	67.0	178.0	1.94	3	24.9
	Mean	23.7	66.4	138.4	1.71	3.2	24.0
Old Normal (43–72 years)							
Male	21	43	64.0	118.0	1.56	2	21.8
	22	70	68.0	141.0	1.76	4	24.6†
	23	44	65.5	125.0	1.62	2	24.2
	24	72	62.0	131.0	1.60	2	20.5
	25	44	62.5	151.0	1.71	2	29.4
	Mean	54.6	64.4	133.2	1.65	2.4	24.1
Female	26	49	60.0	116.5	1.49	2	22.0
	27	62	66.0	123.0	1.62	2	18.5
	28	68	62.0	160.0	1.74	2	21.7
	Mean	59.7	62.7	133.2	1.62	2.0	20.7

* Coefficient of variation of a single observation in the same individual, including day to day variability, is 8.5 per cent.

† Right lung alone: 11.3, 10.9 ml. per min. per mm. Hg.
Left lung alone: 9.4, 9.9 ml. per min. per mm. Hg.

will produce a proportional error in D_L. The duration of breath holding was measured with a precision of about 1 per cent which results in a permissible error in D_L.

Theoretically, the duration of breath holding should not influence D_L, provided the time is measured correctly. However, D_L measured at different breath holding times, from 5 to 14 seconds in Subject 3, is plotted against duration of breath holding in Figure 2 and definitely decreases as duration of breath holding increases. This is in agreement with earlier experiments on the alveolar CO disappearance curve, and is most probably due to the uneven distribution of capillary diffusing surface throughout the lung (1, 15). For this reason a ten-second period of breath holding has been used in these studies. While D_L so obtained may not have the proper absolute value, as a first approximation it should be valid for comparative purposes. The breath holding time is not ex-

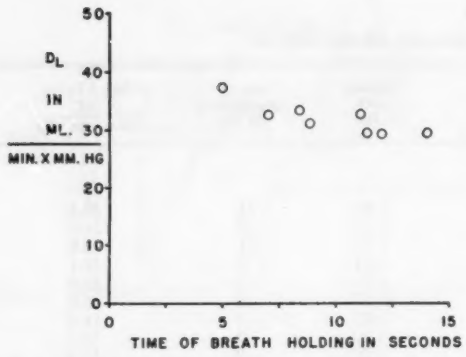

FIG. 2. THE EFFECT UPON CALCULATED D_L OF VARYING THE PERIOD OF BREATH HOLDING

tremely critical; D_L measured at 9 seconds was 1.6 per cent more and at 11 seconds was 1.6 per cent less than its interpolated value at the standard breath holding time of 10 seconds (Figure 2).

The untrained subject takes about 1.5 seconds to inspire the gas mixture containing CO, less than a second to expire the volume of gas needed to wash out his respiratory dead space (750 ml. or more), and between one and two seconds to deliver an adequate (300 ml.) alveolar sample. The period of breath holding was measured from the beginning of inspiration to the beginning of expiration of the alveolar sample (after the dead space wash out) because this approximates the average period the gas molecules were in the lung. If inspiration is prolonged, this estimate of the average time the CO molecules were in the lung will be too great, since much of the CO will not have been in the lung for this period and D_L will be underestimated. When inspiration was slowed to the extent that it occupied over half of the 10-second period of breath holding, D_L decreased a maximum of 13 per cent in Subject 3, falling from a control value of 30.3 to 27.6 and 26.3 ml. per min. per mm. Hg. In addition, inspiration is almost never slowed to this degree in disease states, so that errors from this cause are probably small. If expiration is prolonged, a more common clinical finding, the major effect is to increase the period of collection of the alveolar sample. Since this period is not included in the measured time of breath holding, the latter will in effect be underestimated and D_L will be overestimated. Also, slowing either inspiration and/or expiration will decrease the true average alveolar volume during breath holding to something less than that assumed, *i.e.*, residual volume plus total inspired volume, which will produce an overestimate of true D_L. The actual distortion of D_L depends on the summation of these various effects, and was investigated empirically in two subjects who voluntarily altered their rate of delivery of the expired sample. The time needed to wash out the respiratory dead space was maintained at about 1.0 second representing a flow rate of approximately 750 ml. per sec. or 45 L. per min., which most patients can develop for at least the first part of expiration.

D_L was 30.9, 32.0 and 36.1 ml. per min. per mm. Hg with collection periods of 2.1, 4.5, and 8.1 seconds, respectively, in Subject 3, and 33.3, 37.0, 40.8, and 42.4 ml. per min. per mm. Hg when the collection periods were 1.0, 3.1, 3.6, and 6.5 seconds, respectively, in Subject 6. We concluded that while the duration of the period of collection is not extremely critical, it should be kept less than 2 to 3 seconds, making the total time of expiration, including the time for dead space wash out, less than 3 to 4 seconds. The time for dead space wash out plus sample delivery was measured in nine patients, many of whom had obstructive disease (Patients 1, 2, 11, 12, 17, 19, 22, 25 and 28), without attempting to shorten the time of expiration. In all but one of these patients, an adequate sample was collected in a total expiratory time of less than 3.8 seconds, the remaining patient requiring 4.9 seconds. As a practical matter, the duration of the period of collection of the sample is within the control of the operator, since he starts and stops the collection by turning Tap A (Figure 1). Only a small additional amount of gas is expired after the dead space has been washed out (1 second) and 2 additional seconds of expiration have transpired.

The portion of the expired alveolar gas sampled

The first 750 ml. of each expiration were discarded, as this volume is needed to wash out the dead space gas (16). This makes it difficult to use the present technique in patients with a vital capacity much less than a liter; approximately 750 ml. being used to clear the dead space and approximately 300 ml. being needed for the analyses.

The present technique consists of calculating,

from the dilution of the inspired He concentration, the CO concentration that existed in the expired sample while it was in the lungs and before any CO had been absorbed by the blood. While this is valid in the presence of uneven distribution of inspired gas, the question arises whether the particular alveolar sample collected is representative of the whole lung in regard to diffusing capacity. Evidence has been published (1) that the ratio, pulmonary diffusing capacity/alveolar volume, is not the same throughout the lung. It is also known that the early portions of an expiration are derived from better ventilated alveoli than the later portions (16), so it might be expected that the different parts of the expired breath would come from regions of varying diffusing capacity.

To answer this question, D_L was estimated from samples collected after 1 liter had been expired (early sample) and after 2.5 liters had been expired (late sample) in six normal subjects and in Patients 4 and 6, who had chronic obstructive emphysema. The data are given in Table II. In all but two individuals D_L was higher in the later sample, the average increase being about 10 per cent, a significant change (probability less than 0.05 but greater than 0.01). Some allowance should be made for the fact that expiration took longer and, as discussed above, estimated D_L would increase from this cause alone in those experiments in which late samples were collected. While the data suggest that the later parts of an expiration come from alveoli with slightly greater ratios of D_L to alveolar volume (V_A), the change is not very large and should be investigated further with more precise experiments. We conclude that for clinical use it will probably not make any significant difference as to which part of the expired alveolar gas is used to measure D_L, because the data here, obtained under grossly exaggerated circumstances, show an average change in D_L of only 10 per cent. In many patients there is only sufficient expired volume for one 300-ml. alveolar sample in any case. If a large expired volume is available, the alveolar sample is collected from the second liter.

An interesting question is why, if different parts of the breath contain differing contributions from the 'poorly ventilated' and 'well ventilated' alveoli, and if there is indeed non-uniform distribution of D_L among the different alveoli, is there not more of a difference between the values of D_L obtained from the early and late parts of the breath? The present experiments were performed mainly to ascertain whether this factor is of great importance in making measurements in patients using the present technique and apparatus and not to give a quantitative answer to this problem. However, it should be pointed out that even if inspired gas is distributed unevenly to the different alveoli, if it is distributed without regard to the diffusing capacity of each alveolus, any sample of expired alveolar gas will give the same value of D_L as any other, albeit not the correct total D_L.

Intrathoracic pressure

During the period of breath holding the glottis is normally closed and either the chest is relaxed, being supported by the contained gas, or actually develops expiratory force. In both cases intrathoracic pressure rises, which might impede venous return and decrease cardiac output tending to decrease D_L (2–4, 17, 18). In order to determine the sensitivity of D_L to changes in intrathoracic pressure, simultaneous measurements of D_L and intraesophageal pressure were made in three normal subjects during a Valsalva maneuver. In Subject 3, when intraesophageal pressure rose 37 mm. Hg, D_L did not change from its control value of 29.4 ml. per min. per mm. Hg. In Subject 6 when intraesophageal pressure rose 64 mm. Hg, D_L de-

TABLE II

Effect upon D_L of sampling different parts of the expired alveolar gas

Subject	Diagnosis	D_L $\frac{ml.}{min. \times mm.\ Hg}$ Early alveolar sample*	D_L $\frac{ml.}{min. \times mm.\ Hg}$ Late alveolar sample†
1	Normal	22.6	25.1
3	Normal	36.9	38.6
4	Normal	31.0	30.3
9	Normal	38.3	44.6
12	Normal	18.8	17.8
22	Normal	24.6	29.2
Pt. 4	Emphysema	20.5	22.6
Pt. 6	Emphysema	32.3	34.2
	Mean	27.5	30.3

* Early sample: mean volume of 1.0 liters expired before sampling.

† Late sample: mean volume of 2.5 liters expired before sampling.

creased from 37.4 to 33.2 ml. per min. per mm. Hg and in ABD, one of our colleagues, when intraesophageal pressure rose 44 mm. Hg, D_L decreased from 36.7 to 30.5 ml. per min. per mm. Hg. Since, under these extreme conditions, D_L decreased a maximum of 17 per cent, it is unlikely that the variations in intrathoracic pressure normally encountered during breath holding would be a major cause of changes in D_L.

As stated in the discussion of the variations in D_L with body position (*vide infra*), a 100 per cent increase in cardiac output during exercise is associated with about a 30 per cent increase in D_L. We assume that the changes in intrathoracic pressure in these experiments were either not great enough or not of sufficient duration to produce changes in cardiac output of this magnitude.

Effects of variation in lung volume

Krogh (9) reported that D_L increased proportionally to alveolar volume above midcapacity which if true would necessitate correcting the measured values of D_L to a standard volume in the manner she did. However, other work from this laboratory (19) suggests that D_L does not change markedly with lung volume, but rather that Krogh's results were exaggerated by the uneven distribution of inspired gas. In order to clarify this particular point for the exact conditions of the test, D_L was measured at different alveolar volumes in five normal subjects and the data are presented in Table III. When alveolar volume increased an average of 56 per cent, D_L increased an average of only 9 per cent, while according to Krogh's data the increase should have been approximately 50 per cent. For this reason we have made no corrections for differences in lung volume.

Position of the body

The D_L of seven healthy subjects was measured in the supine, sitting and (except for one subject) standing positions. The results are presented in Table IV. The appropriate position was adopted for five minutes before each test. D_L was greater in the supine than in the sitting position in all seven subjects (a significant change; probability less than 0.01) and higher in the sitting than in the standing position in five out of six subjects (not a significant change; probability 0.11). Since the values for residual volume used in the calculation of D_L were measured in the sitting position, the significance of the changes in D_L depends on the constancy of the residual volume with changes in position. Therefore, the original calculations of D_L have been 'corrected' assuming a 20 per cent decrease in residual volume on changing from a sitting to a standing position and a 30 per cent increase on changing from a sitting to a supine position, the largest changes in residual volume reported in the literature (20, 21). When the corrections were applied, D_L was still found to be greater in the supine than in the sitting position in six out of seven subjects, but there was no longer a significant alteration on changing from sitting to standing. We measured the residual volume of Subject 9 (whose D_L decreased most on standing) by the method of Darling, Cournand, and Richards (11) in the sitting, standing, and supine positions and found no significant change (1168, 1250, and 1178 ml. BTPS, respectively). We concluded

TABLE III

Dependence of D_L on alveolar volume

Subject	Small alveolar volume*		Large alveolar volume*		Change in D_L %
	Alveolar volume *ml. STPD*	D_L *ml. / min. × mm. Hg*	Alveolar volume *ml. STPD*	D_L *ml. / min. × mm. Hg*	
3	2,980	28.4	4,700	32.1	+13
4	3,240	31.2	5,060	33.1	+ 6
9	3,260	42.8	5,160	36.1	−16
12	2,385	16.1	3,490	19.0	+18
14	2,910	23.8	4,690	29.6	+24
				Average change	+ 9

* Data are single values of D_L.

TABLE IV

The effect of change in position upon D_L

Subject	Sitting	Supine		Standing	
		"Uncorrected"	"Corrected"*	"Uncorrected"	"Corrected"*
	D_L $\frac{ml.}{min. \times mm.\ Hg}$				
1	25.4	30.5	28.8	22.5	23.8
3	30.7	34.4	32.8	29.8	32.2
4	31.2	37.0	34.2	25.7	28.9
9	37.7	40.6	38.1	30.6	33.3
12	17.2	20.7	18.9	18.2	20.1
13	36.6	43.0	40.0		
14	31.3	32.4	30.2	29.9	32.2
Mean	30.0	34.1	31.8	26.2	28.4

* D_L calculated on the assumption that the change from the sitting to supine position produces a 20 per cent reduction in residual volume and from sitting to standing a 30 per cent increase in residual volume.

that D_L was probably greater in the lying than in the sitting position, and possibly greater in the sitting than in the standing position, and, for purposes of comparison, have made all measurements of D_L sitting. The possibility that changes in cardiac output alone caused the observed changes in D_L in the different positions was unlikely for the following reasons. In a recent study by Donald, Bishop, Cumming, and Wade (22) cardiac output was found to increase 6 to 7 per cent on changing from the sitting to the supine position. According to other data of this same group (23) cardiac output breathing air would approximately double when O_2 consumption was increased to 2000 ml. per min. from rest. According to the data in Figure 4, relating D_L and O_2 consumption during exercise, a 100 per cent increase in cardiac output produces about a 30 per cent increase in D_L. If this relationship were linear the effect of a 6 per cent increase in cardiac output would be hardly measurable.

Variation in D_L with alveolar O_2 tension

D_L decreases as alveolar O_2 tension is raised (1) presumably because the resultant rise in capillary O_2 tension decreases the rate of the combination of CO with intracorpuscular hemoglobin (6, 7) (see Footnote 6). D_L measured while breathing

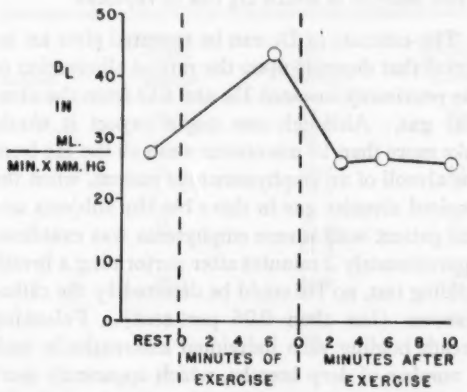

FIG. 3. THE RECOVERY OF D_L FOLLOWING EXERCISE

The exercise consisted of walking at 4½ miles per hour on an 8 per cent gradient and corresponded to an O_2 consumption of about 2500 ml. per min.

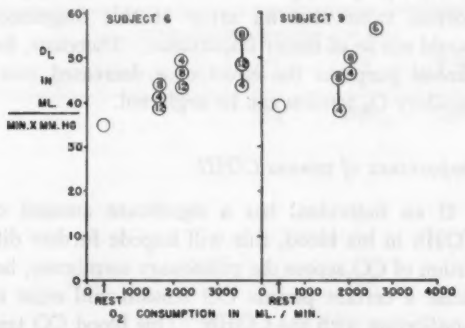

FIG. 4. D_L IN RELATION TO O_2 CONSUMPTION DURING EXERCISE

The figures within the circles indicate the duration of exercise in minutes at the time of measurement. The measurements were made at rest, and during exercise at 3, 4, and approximately 5 miles per hour on an 8 per cent gradient.

approximately 100 per cent O_2 may be as low as half its value breathing air (24). Similarly, if the mean capillary O_2 tension during the test is below its usual value of 120 mm. Hg, D_L will be overestimated. Over a range of mean capillary O_2 tension from 40 to 200 mm. Hg the correct D_L equals measured $D_L \times (0.70 + 0.0027$ mean capillary O_2 tension in mm. Hg) (24). While a high mean capillary O_2 tension can be avoided by keeping the inspired O_2 concentration at about 21 per cent, a low mean capillary O_2 tension can occur in disease where there is interference with the oxygenation of the capillary blood. It is of course the mean capillary and not the arterial O_2 tension which is of importance in affecting the rate of CO uptake by the red cells. In the data presented in this paper, no correction has been made for changes in capillary O_2 tension for two reasons. (a) From a practical standpoint, patients with pulmonary disease will tend to have mean capillary O_2 tensions less than 120 mm. Hg during the period of breath holding, which will artificially increase D_L. Therefore a patient with a reduced D_L most probably has impaired diffusion, although it may be more serious than consideration of the measured value of D_L would indicate. (b) It is unlikely that the mean capillary O_2 tension in the alveoli which contribute the expired alveolar sample will ever drop below 40 mm. Hg, in which case according to the relationship given above, D_L would be overestimated by 19 per cent. However, in this situation the proper D_L would be so reduced below its normal value that an error of this magnitude would not be of major importance. Therefore, for clinical purposes the effect of a decreased mean capillary O_2 tension can be neglected.

Importance of venous COHb

If an individual has a significant amount of COHb in his blood, this will impede further diffusion of CO across the pulmonary membrane, because a certain plasma CO tension will exist *in equilibrium* with the COHb. This blood CO tension can be calculated from the Haldane relation,

$$\frac{210 \text{ CO tension (or gas concentration)}}{O_2 \text{ tension (or gas concentration)}} = \frac{\text{COHb concentration}}{O_2\text{Hb concentration}} \quad (3)$$

with the proviso that no large amounts of reduced Hb are present (15). In an extreme case, such as that of a heavy smoker who has as much as 10 per cent COHb in his blood (25), the equilibrated plasma CO tension would be less than

$$\frac{10 \text{ per cent COHb}}{90 \text{ per cent } O_2\text{Hb}} \times \frac{17.5 \text{ per cent } O_2}{210}$$

or 0.0092 per cent CO,

assuming the capillary O_2 tension equal to alveolar O_2 tension (125 mm. Hg or 17.5 per cent O_2) and capillary O_2Hb equal to arterial O_2Hb. This equilibrated blood CO concentration (0.0092 per cent) should be subtracted from both the initial and final CO concentrations in the expired alveolar sample (15). If the subject had an alveolar volume of 6 liters, an initial CO concentration of 0.15 per cent, and a true D_L of 30 ml. per min. per mm. Hg, his alveolar CO concentration would be 0.087 per cent CO at the end of 10 seconds. If one ignored the presence of the COHb in the blood, D_L would apparently be 27.4 ml. per min. per mm. Hg, an error of 8 per cent. Since this is an extreme case and since it would add greatly to the complexity of the test to measure blood COHb, we have not estimated blood COHb in each individual. However, all individuals were questioned about their smoking habits, and an effort was made to keep the initial CO concentration high enough to minimize this error.

Time interval at which D_L can be repeated

The estimate of D_L can be repeated after an interval that depends upon the rate of elimination of the previously inspired He and CO from the alveolar gas. Although one might expect it would take more than 10 minutes to wash all the He from the alveoli of an emphysematous patient, when the expired alveolar gas in three healthy subjects and one patient with severe emphysema was examined approximately 2 minutes after performing a breath holding test, no He could be detected by the catharometer (less than 0.05 per cent). Following breath holding each individual automatically took a number of deep breaths, which apparently were sufficient to wash out the He. During exercise studies, it would be expected that the He would be washed out even faster. CO will disappear from the alveoli at a considerably faster rate than

the He since it is absorbed by the blood in addition to being washed out by inspired gas. Therefore, we considered it permissible to repeat measurements of D_L after 2 minutes.

There will be an increase in blood COHb with each estimation of D_L but calculation shows this to be small. For instance, if an individual starts the breath holding period with an alveolar volume of 6 liters and an alveolar CO concentration of 0.15 per cent, making a total of 9 ml. of CO, and absorbed *all* of this into a total blood CO capacity of 1200 ml., his blood COHb concentration would rise 0.75 per cent. According to the Haldane relation, under the above conditions the resulting increase in equilibrated gas CO concentration would be less than

$$\frac{0.75 \text{ per cent COHb}}{98 \text{ per cent } O_2Hb} \times \frac{17.5 \text{ per cent } O_2}{210} = 0.0006 \text{ per cent CO}$$

which can be neglected. Therefore, several measurements of D_L can be made in succession without increasing the blood COHb by a significant amount. This has been verified experimentally (1).

Present evidence suggests that no significant alterations in the pulmonary capillaries are produced by the respiratory maneuvers inherent in the present technique (1). Since D_L returns to normal several minutes after heavy exercise (*vide infra*), any changes in the pulmonary capillaries resulting from the effort of one estimation would certainly have subsided within several minutes.

Effect of exercise on D_L

The increase in D_L[10] during five minutes of exercise on an 8 per cent gradient at 4½ m.p.h. in five normal subjects is shown in Table V, and ranged from 42 to 58 per cent (mean of 45.8 per cent). Also shown in Table V are measurements made during the period of recovery from exercise in three subjects, which indicate that D_L returns to resting control values within two to three minutes of the cessation of exercise. In the instance of Subject 6 several subsequent minutes of exercise occurred after the value of 43.9 ml. per min. per mm. Hg was obtained, which probably explains the higher value of 47.1 ml. per min. per mm. Hg during the recovery period. The rapidity of the changes in D_L are illustrated in Figure 3, a graph of the findings in Subject 1 before, during and after exercise.

An increase in D_L with exercise was first reported by Krogh (9) who found an average 36 per cent rise above the resting level by the breath holding technique, not significantly different from

[10] Residual volume determined at rest was used to calculate D_L.

TABLE V

Changes in D_L during and after exercise

Subject	D_L* Sitting before exercise	D_L*,† During exercise	Mean % increase in D_L during exercise	D_L* Sitting after cessation of exercise				
				½ minute	1 minute	2–3 minutes	5–6 minutes	9–10 minutes
1	26.8	42.4 42.2	58			25.4	26.0	24.8
3	36.8	52.4 52.5	42					
4	31.2	41.4 47.8	43					
6	30.9	43.9	42	47.1		33.5	33.6	
9	38.2	60.6 49.3	44					
12	18.6				24.6	20.4	20.9	19.7

* D_L is in $\frac{\text{ml.}}{\text{min.} \times \text{mm. Hg}}$.

† Measurements were made between the fourth and sixth minutes of walking on a treadmill at 4½ m.p.h. on an 8 per cent gradient.

our average increase of 46 per cent with a modification of her method. On the other hand $D_{L_{O_2}}$ has been reported as increasing 300 per cent with exercise (3), D_L by the method of Filley, MacIntosh, and Wright as increasing 114 per cent (4) and by the method of Bates, Boucot, and Dormer, 75 to 80 per cent (17). The grade and duration of exercise were not always comparable but since the presently reported O_2 uptakes during exercise are as large or larger than those reported during steady state measurements of D_L and $D_{L_{O_2}}$ (4, 26, 27, 28), it seems apparent that estimates of resting D_L by steady state methods are less than those by breath holding techniques (see section C) and increase proportionally more with exercise. There are many theoretical and practical differences between the steady state and breath holding methods which might explain the different absolute values of D_L (and $D_{L_{O_2}}$), but as a first approximation, each method should be consistent within itself implying that the proportional increase in D_L from rest to exercise should be the same for all. There is no adequate explanation for the discrepancy at this time. However there is no reason to suspect that the theoretical relation of D_L to $D_{L_{O_2}}$, namely, $D_{L_{O_2}} = 1.23\ D_L$ (9), is grossly in error. Although the "breath holding D_L" is measured at a larger lung volume than the "steady state D_L," this should not produce a large change (19). All present methods underestimate the true diffusing capacity of the pulmonary membrane because they ignore the finite velocity of the uptake of CO or O_2 by the corpuscles (5).

The effect upon D_L of varying the grade and duration of exercise in Subjects 6 and 9 is recorded in Figure 4. Both subjects were exercised at 3, 4, and 5 m.p.h. at a slope of 8 per cent and D_L was measured after 4, 8, and in the case of Subject 6, 12 minutes on the treadmill. O_2 consumption was measured over half-minute periods between the D_L estimations, and was reasonably stable at a given grade of work ($\pm$ 10 per cent). The values of D_L in Figure 4 have been plotted against the average O_2 consumption at each grade of exertion. It can be seen that with each increase in O_2 consumption, there was some further increase in D_L in both subjects. Riley, Shepard, Cohn, Carroll, and Armstrong (28) have reported that $D_{L_{O_2}}$ reaches a maximum plateau with increasing exercise if it is plotted against O_2 consumption, and have used the maximal $D_{L_{O_2}}$ as an index of the total pulmonary capillary surface. The interest in this maximal measurement is based primarily on the expectation that abnormalities which are undetectable in the resting state will become more apparent under the stress of exercise, and secondarily on the fact that the measurement of $D_{L_{O_2}}$ is more precise under these conditions. The question of whether a true maximal diffusing capacity is reached in all subjects is pertinent. $D_{L_{O_2}}$ tends to increase with decreasing arterial O_2 tension, other things remaining constant (28). Therefore, in measurements of maximal $D_{L_{O_2}}$ in which both exercise and anoxia are present there is the distinct possibility that these two factors act to produce an apparent plateau of $D_{L_{O_2}}$ when it is plotted against O_2 consumption. While this criticism does not apply to the steady state measurements of D_L, only two unequivocal 'plateaux' with increasing exertion were reported (4). Bates, Boucot, and Dormer (17), measuring D_L by a steady state and tidal sampling method, found that of those subjects in whom the presence or absence of a plateau could be fairly assessed, about half showed an increase as the estimated O_2 consumption rose from 1.2 to 2.2 liters per min. and half did not. In two subjects who were studied in more detail and whose O_2 consumptions were measured, D_L could not be said to have reached a plateau. The two subjects studied in detail by the breath holding method in the present report did not reach a plateau (see Figure 4). Ideally, this problem should be investigated by measuring D_L or $D_{L_{O_2}}$ during different grades of exercise and as a function of pulmonary blood flow.

In addition to the above studies on D_L which suggest that small amounts of exertion are without effect, D_L was measured in two of the healthy subjects immediately after they had been walking around the room and was found to be the same as it was after they had been quietly seated for 30 minutes. Therefore, several minutes rest in a chair prior to the measurement of D_L seemed sufficient to provide a standardized condition of activity.

Effect of hyperventilation

Patients sometimes become apprehensive in the presence of unfamiliar personnel and equipment,

and hyperventilate. As this might be expected to alter D_L, it was measured immediately before and after a period of voluntary hyperventilation, in three normal subjects (1, 6, and 19). The average control value was 30.9, the average value after hyperventilation was 30.0 ml. per min. per mm. Hg. Although Subject 6 hyperventilated for five minutes, reducing his expired alveolar CO_2 concentration from 4.9 to 2.6 per cent, his D_L only went from 34.8 to 33.7 ml. per min. per mm. Hg. Therefore it appears that hyperventilation is not an important factor to control in standardizing the measurement of D_L.

In summary, if the measures which have been discussed are used to standardize the procedure, the theoretical cumulative variation in D_L among individuals caused by these various factors could be as high as roughly 30 per cent. In actuality many of these influences cancel out or are constant in a given individual, so that the actual variation in estimated D_L in a single individual is very much less than 30 per cent. In fact, as reported below, the coefficient of variation in a normal subject is only 8.5 per cent. The absolute error in D_L is unknown at present; the greatest usefulness of the measurement lies in comparisons.

B. *Reproducibility of D_L*

In order to determine its reproducibility the values of D_L in 24 healthy subjects and 19 patients

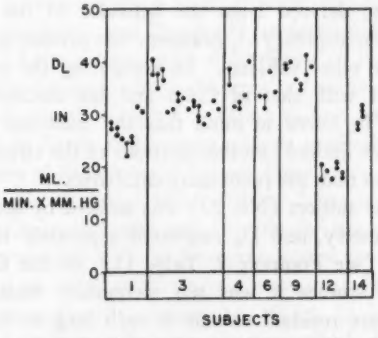

FIG. 5. REPEATED ESTIMATIONS OF RESTING D_L IN SEVEN HEALTHY SUBJECTS

Different days of observation are plotted at different points on the abscissa, progressing from left to right. Observations which are plotted at the same value of the abscissa were obtained on the same day. The longest overall period of observation was seven months, in Subject 4; the shortest was two weeks, in Subject 14.

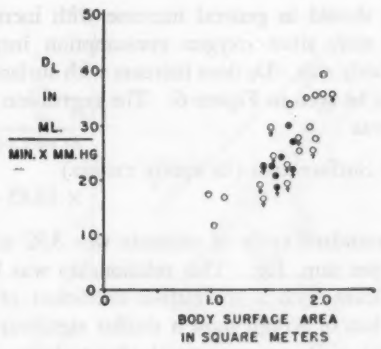

FIG. 6. RESTING D_L IN HEALTHY SUBJECTS PLOTTED AGAINST BODY SURFACE AREA

○ male subjects under 35 years of age; ♀ female subjects under 35 years of age; ● male subjects over 40 years of age; ♀ female subjects over 40 years of age.

with various lung diseases in whom two or more estimations had been made the same day, were analyzed statistically. The coefficient of variation for a single measurement on the same day in a normal subject was 5.8 per cent, and in a patient, 7.4 per cent. These statistics are not significantly different (probability greater than 0.05). In addition, statistical variations in D_L were analyzed in seven healthy subjects who had been studied on from 2 to 14 different days over periods ranging from 2 to 28 weeks in order to assess the variation in D_L from one day to the next. The data are plotted in Figure 5. The coefficient of variation, including variation both between and within days, was 8.5 per cent. For practical purposes this means that a single estimate of D_L on a given subject has a 76 per cent chance of lying within $\pm$ 10 per cent of the true mean value. An analysis of variance was performed on the data: the variation between days had a coefficient of variation of 9.4 per cent, that within a day, 5.8 per cent. These statistics were significantly different; the variation between days was significantly greater than that within a day (probability less than 0.01).

C. *D_L in Healthy Subjects and Its Relation to Body Size, Sex and Age*

The vital statistics and mean D_L for each of the 28 healthy subjects are given in Table I. The mean D_L was 24.9 ml. per min. per mm. Hg with a range from 11.0 to 37.5 ml. per min. per mm. Hg.

D_L should in general increase with increasing body size, since oxygen consumption increases with body size. D_L does increase with surface area as can be seen in Figure 6. The regression equation was

D_L = Surface area (in square meters) $\times$ 18.85 − 6.8.

The standard error of estimate was 3.92 ml. per min. per mm. Hg. This relationship was highly significant with a correlation coefficient of 0.81. The data of Krogh show a similar significant correlation of D_L, as measured by her technique, with surface area (29). D_L as measured by a steady end tidal sampling technique does not show a correlation with body size at rest, but does show a correlation with functional residual capacity during exercise, which may reflect body size (17). The practical importance of the present findings is that any attempt to predict a normal D_L for a given patient must take body size into account; D_L almost doubles over the range of normal adult surface area (see Figure 6). There is a question as to what is the best index of body size. We have chosen surface area, mainly because of its known relation to O_2 consumption. However, D_L is also significantly correlated with height and weight. The regression equation for D_L as a function of height is D_L = height (in inches) $\times$ 0.874 − 31.6. The correlation coefficient is 0.74. The regression equation of D_L as a function of weight is D_L = weight (in pounds) $\times$ 0.149 + 5.2. The correlation coefficient was 0.78. There is no significant difference among the correlation coefficients for D_L with surface area, weight or height, so there is no statistical reason for choosing among them at this time. It should be noted that since the regression line of D_L as a function of surface area does not pass through the origin, simply dividing D_L by surface area does not produce an index independent of surface area. Cohn, Carroll, Armstrong, Shepard, and Riley (27) found a regression coefficient of 1.7 for 'maximal' D_{Lo_2} as a function of height in inches, but since these measurements were taken during exercise, while our measurements were made at rest, the difference is not unexpected. Kruhøffer (30) also reports an increase in D_L with height.

The average resting value of D_L in normal subjects, 24.9 ml. per min. per mm. Hg, by the present breath holding technique is not significantly different from the results of Krogh (9) and Bøje (18) using a similar technique. However, it is larger than the comparable values of 16.9 and 17.6 ml. per min. per mm. Hg for the steady state methods of Filley, MacIntosh, and Wright (4) and Bates, Boucot, and Dormer (17), respectively, as well as being greater than the lower limit of the normal D_{Lo_2}, *i.e.*, 15 ml. per min. per mm. Hg (26). While there are many possible explanations for the discrepancies, some of which have been mentioned in the discussion of the effects of exercise or in earlier publications (1, 15), this problem should be investigated further.

Although estimates of D_L by the present method tend to be lower in women than in men (Figure 6) there was no significant difference between the sexes in the present limited data.

No correlation was found between the resting D_L and age. Cohn, Carroll, Armstrong, Shepard, and Riley (27) reported a significant decrease in maximal D_{Lo_2} with increasing age, although there is doubt as to whether a maximum had been reached by some of the older subjects who were unable to exercise strenuously. In their studies direct evidence of maximality (no significant change in D_{Lo_2} at two different levels of oxygen consumption) is available in only two of the eight subjects over 45 years of age and one of these actually showed the highest value for D_{Lo_2} in the entire group. However, indirect evidence of maximality, derived from the behavior of the mean alveolar-capillary O_2 gradient, was present in some of the other subjects. In comparing the present report with that of Cohn and his associates, it must be borne in mind that the 'maximal D_{Lo_2}' may be limited by other portions of the circulatory system than the pulmonary capillaries.

One subject (No. 22) was studied by bronchospirometry, and D_L measured separately in each lung (see Footnote 2, Table I). At the time of these studies it was not technically feasible to measure residual volume in each lung so that residual volume estimates for the whole lung were distributed in the ratio of 55 per cent on the right side to 45 per cent on the left side (31). While the agreement between D_L for the whole lung (24.6 ml. per min. per mm. Hg) and the sum of D_L for the two lungs (11.1 + 9.6 = 20.7 ml. per min. per mm. Hg) is quite good, it is interesting

that in this healthy subject, as well as in four patients (see Table VI), D_L for both lungs together is greater than the sum of the D_L's for the two lungs separately. The distribution of D_L between the two sides in the normal subject is approximately in proportion to their relative alveolar volumes.

D. *Measurements of D_L in Patients*

The vital statistics, results of pulmonary function tests and mean D_L, as well as the predicted normal range of D_L, for the 28 patients are presented in Table VI. The values for D_L in the patients are plotted against body surface area in Figure 7 which also contains the normal regression boundaries for ± twice the standard error of estimate.

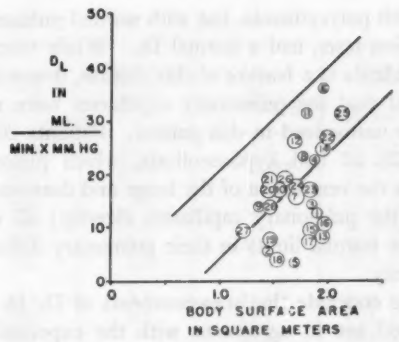

FIG. 7. RESTING D_L IN 28 PATIENTS PLOTTED AGAINST BODY SURFACE AREA

The numbers inside the circles indicate the patient recorded in Table VI. The parallel lines are ± twice the standard error of estimate about the regression line.

D_L is theoretically dependent on 1) the surface area of the pulmonary capillaries in contact with alveolar gas, 2) the thickness of the pulmonary membrane, and 3) the specific resistance to gas diffusion of the tissue making up the membrane. At present these factors cannot be separated. However, in general we would expect D_L to be less than normal in any condition in which the number or size of the pulmonary capillaries is decreased, the thickness of the pulmonary membrane increased, or in which the tissue composing the pulmonary membrane is less permeable to gas diffusion. In chronic obstructive emphysema, there is destruction of alveolar septa and pulmonary capillaries, and D_L should be decreased (32–34). Of the patients with emphysema (1 through 7), all but two did have abnormally low values of D_L. Although Krogh (9) failed to find any decrease in D_L in emphysema, this is explicable on the basis of the effects of uneven distribution of inspired gas on her results. Patient 2 had a much larger D_L on the right lung than on the left, which was compatible with the interpretation of the x-ray films of his chest which showed more gross bullous changes on the left. Patients 8, 10, 11, 12, 14 and 15 had various pulmonary diseases which do not attack the capillaries primarily, but in which capillary damage is secondary to destruction or displacement of regions of the lung. The effect on D_L would therefore be expected to depend on the amount of parenchymal tissue lost; in this group, variations in D_L were in fact commensurate with the severity and extent of the lesions. Patient 8 had a larger D_L in the left lung than in the right, which was compatible with the large amount of tissue displaced by cyst in his right lung. Patient 11 had fibrocavernous tuberculosis in the upper lobe of his right lung which would be expected to decrease the D_L in the right lung, as it did. Patient 9 who had bronchial asthma, a disease which does not primarily involve the capillaries (35), had a normal D_L, while Patients 16, 17, 18 and 19 with diseases which are known to attack the pulmonary capillaries, all had very low estimates of D_L (36). Since Patients 20 and 21 both had pulmonary hypertension with no findings suggesting a congenital cardiac defect, and since in addition both had normal values of D_L, we concluded that their pulmonary disease had affected the pulmonary arteries and arterioles, but not the pulmonary capillaries. Patient 22 with fibroendocarditis and Patient 23 with mitral stenosis were not in congestive heart failure at the time of the studies, and so it is not unreasonable to find that their pulmonary diffusing capacities are little affected. One would conclude that they had no significant pulmonary capillary damage. Both Patients 13 and 24 had a decreased pulmonary vascular bed, the former because of pneumonectomy, and the latter because of obstruction of the pulmonary artery to the right lung. The D_L on the obstructed side in Patient 24 was slightly more than half that of the contralateral lung. As would be expected, both patients had decreased total D_L estimates. Patient

25 with polycythemia, but with normal pulmonary function tests, had a normal D_L. While vascular thrombosis is a feature of this disease, it was concluded that the pulmonary capillaries were relatively uninvolved in this patient. Patients 26, 27 and 28 all had kyphoscoliosis, which primarily limits the ventilation of the lungs and does not attack the pulmonary capillaries directly; all were within normal limits in their pulmonary diffusing capacity.

We conclude that measurements of D_L by this method are in agreement with the expected derangements in pulmonary physiology. From the practical point of view, the great advantages of the method are that it takes so little of the time, requires no arterial samples, and can be repeated often without discomfort to the patient.

SUMMARY

A modification of the Krogh breath holding technique for the clinical measurement of apparent pulmonary diffusing capacity (D_L) is described. Reproducibility was investigated in 28 normal subjects; the coefficient of variation for a single meas-

TABLE VI

D_L and pulmonary function tests in patients with various disorders of the chest *,†*

Patient number	Diagnosis	Sex	Age *yrs.*	Height *inches*	Weight *pounds*	Surface area M^2	VC *ml.*	IC *ml.*	ERV *ml.*	RV *ml.*
1	Chr. obstr. emphysema	M	55	71.5	158	1.91	2840 (3900)	1530 (3120)	1340 (780)	3740 (1730)
2	Chr. obstr. emphysema	M	57	64	101	1.45	1760 (3450)	1170 (2760)	580 (690)	2740 (1520)
3	Chr. obstr. emphysema	M	47	67	165	1.86	2680 (3800)	1990 (3040)	680 (760)	3260 (1160)
4	Chr. obstr. emphysema	M	75	67	165	1.86	2980 (3270)	1800 (2620)	1210 (650)	2950 (1450)
5	Chr. obstr. emphysema	M	57	69	122	1.68	2000 (3720)	1150 (2980)	920 (740)	4320 (1640)
6	Chr. obstr. emphysema	M	55	70	165	1.96	3260 (3820)	1950 (3060)	1420 (760)	3880 (1680)
7	Localized obstr. emphysema, right lung	M	28	66	135	1.69	2000 (4200)	1420 (3360)	890 (840)	1700 (1050)
8	Solitary lung cysts	M	27	73	142	1.84	3750 (4560)	2550 (3650)	1500 (910)	1870 (1140)
9	Bronchial asthma	F	14	61	95	1.37	1690 (2600)	1360 (2080)	620 (520)	1290 (650)
10	Bronchiectasis and broncho-pleuro-cutaneous fistula	M	54	66.5	120	1.58	1820 (3650)	520 (2920)	910 (730)	2550 (1610)
11	Fibro-cavernous tuberculosis (right upper lobe)	M	60	72	148	1.80	3140 (3820)	1670 (3060)	1600 (760)	3110 (1680)
12	Bronchial carcinoma	M	52	66	135	1.70	2480 (3650)	1730 (2920)	750 (730)	1520 (1610)
13	Postpneumonectomy for bronchial carcinoma	M	55	72	134	1.80	3080 (3930)	1800 (3140)	1150 (790)	2690 (1450)
14	Chemical burn of respiratory tract	M	30	72	162	1.95	3250 (4400)	2550 (3520)	1000 (880)	2380 (1100)
15	Chemical burn of respiratory tract	M	44	67	164	1.84	1970 (3860)	880 (3100)	1110 (760)	1870 (1190)
16	Diffuse interstitial fibrosis	F	35	61	220	1.97	2600 (2830)	1800 (2260)	740 (570)	2060 (870)
17	Diffuse interstitial fibrosis	F	52	67.5	153	1.81	1500 (2830)	780 (2260)	560 (570)	740 (1270)
18	Sarcoidosis	M	53	62	119	1.53	2860 (3400)	1780 (2720)	1360 (680)	1570 (1500)
19	Scleroderma	M	45	62	107	1.46	1780 (3550)	960 (2840)	720 (710)	850 (1100)
20	Pulmonary arteriolar disease	M	15	66	101	1.50	2050 (3140)	1500 (2520)	660 (620)	1390 (780)
21	Pulmonary arteriolar disease	F	44	62.5	106	1.46	2760 (2760)	1740 (2200)	1120 (560)	1520 (840)
22	Fibroendocarditis	M	31	72	169	1.97	4150 (4400)	3100 (3520)	1050 (880)	1920 (1100)
23	Mitral stenosis	M	28	70.5	137	1.80	3300 (4380)	2260 (3500)	980 (880)	1670 (1120)
24	Obstructed right pulmonary artery	M	50	64.5	145	1.71	3880 (3620)	2670 (2900)	1780 (720)	1740 (1580)
25	Polycythemia vera	M	39	72	200	2.13	6300 (4250)	4500 (3400)	1640 (850)	3280 (1300)
26	Kyphoscoliosis	F	17	67	111	1.58	1920 (2940)	1600 (2350)	260 (590)	1080 (760)
27	Kyphoscoliosis	F	46	50	99	1.22	950 (2180)	650 (1750)	260 (430)	750 (670)
28	Kyphoscoliosis	M	30	61	102	1.41	1050 (3750)	820 (3000)	420 (750)	900 (950)

* *Symbols:* VC equals vital capacity; IC equals inspiratory capacity; ERV equals expiratory reserve volume; RV equals residual volume; TLC equals total lung capacity; MBC equals maximal breathing capacity; all lung volumes BTPS.

† Figures in parentheses are predicted normal values.

‡ RV/TLC in young normals should be less than 30 per cent.

§ Maximum inspiratory (or expiratory) velocities are measured between 200 and 1200 ml. of the inspiration (or expiration). Normal values lie between 400 and 600 liters per min.

TABLE VI—*Continued*

Patient number	TLC *ml.*	RV/TLC‡ ×100	MBC *L./min./M²*	Max. exp. vel.§ *L./min.*	Max. insp. vel.§ *L./min.*	N_2 single breath test‖ *% N_2*	D_L *ml./min.×mm. Hg*	Normal¶ range of D_L *ml./min.×mm. Hg*
1	6580 (5630)	57		37	120	12.0	12.2	21.0–37.0
2	4500 (4970)	60	28 (44)	28	220	13.5	6.5**	12.7–28.7
3	5940 (4960)	55		50	280	4.6	13.6	20.0–36.0
4	5930 (4720)	50	30 (47)	50	220	4.0	21.6	20.2–36.2
5	6320 (5360)	68	18 (57)	15	150	8.0	3.5	17.1–33.1
6	7140 (5500)	54	27 (58)	105	170	6.4	35.2	22.0–38.0
7	3700 (5250)	46	44 (72)	76	122	1.5	15.8	17.2–33.2
8	3620 (5700)	33	53 (72)	210	165	3.7	22.4††	19.9–35.9
9	3980 (3250)	32	39 (64)	53	100	0.8	14.1	11.1–27.1
10	4370 (5260)	58	44 (58)	70	135		16.7	15.2–31.2
11	6250 (5500)	50	35 (46)	138	210	4.0	31.1‡‡	19.2–35.2
12	4000 (5260)	38		150	220	1.8	25.6	17.3–33.3
13	5770 (5380)	47	24 (58)	65	220	5.7	8.6	19.2–35.2
14	5630 (5500)	42	26 (70)	115	130	2.2	24.2	22.0–38.0
15	3840 (5050)	48	36 (64)	50	45	4.0	9.7	19.9–35.9
16	4660 (3700)	44	36 (55)	68	200	6.6	10.2	22.2–38.2
17	2240 (4100)	33	49 (47)	200	158	9.8	7.1	19.3–35.3
18	4430 (4900)	35		200	170	8.2	4.0	14.2–30.2
19	2630 (4650)	32	80 (63)	430	250	5.3	7.0	12.8–28.8
20	3340 (3920)	42	64 (79)	140	250	1.7	17.1	13.5–29.5
21	4280 (3600)	35	65 (50)	160	140	0.6	18.7	12.8–28.8
22	6070 (5500)	32		600	370	0.7	26.3	22.1–38.1
23	4970 (5500)	33	45 (72)	300	270	2.5	17.1	19.1–35.1
24	5620 (5200)	31	44 (60)	325	180	1.5	17.6§§	17.4–33.4
25	9580 (5550)	34	117 (66)	800	550	1.7	30.4	24.9–40.9
26	3000 (3700)	36	38 (63)	122	140	3.0	17.5	15.2–31.2
27	1700 (2850)	44	27 (50)	44	89	2.0	9.4	8.1–24.1
28	1950 (4700)	41	30 (71)	77	75		13.3	11.8–27.8

‖ The change in N_2 concentration between 750 ml. and 1250 ml. of the expired air should be less than 2.0 per cent.
¶ ± twice the standard error of estimate about the regression line.
** Patient 2: D_L right lung was 3.4 ml./min./mm. Hg; left lung 2.0 ml./min./mm. Hg.
†† Patient 8: D_L right lung was 5.6 ml./min./mm. Hg; left lung 10.8 ml./min./mm. Hg.
‡‡ Patient 11: D_L right lung was 10.5 ml./min./mm. Hg; left lung 17.5 ml./min./mm. Hg.
§§ Patient 24: D_L right lung was 4.4 ml./min./mm. Hg; left lung 8.8 ml./min./mm. Hg.

urement on a single subject was 8.5 per cent. D_L increased with increasing body surface area, height and weight. It also increased with exercise, returning to control values within several minutes of the cessation of exercise. D_L rose with increasing degrees of exercise, but did not reach a maximal plateau in the subjects studied. D_L varied slightly with posture, being greatest when the subject was supine, less when sitting and least when standing. The importance of these and other factors in obtaining a standardized estimate of D_L is discussed.

Measurements of D_L were made in 28 patients with various pulmonary diseases. In four patients and one normal subject, D_L was measured separately in the two lungs. There were no difficulties in performing the test, and the values of D_L obtained were consonant with the clinico-pathological diagnosis.

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APPENDIX

Patients 1 through 6 had histories and physical and x-ray findings compatible with a diagnosis of chronic obstructive emphysema, which was confirmed by the pulmonary function tests. Patient 7 was a very much younger man giving a similar history, but x-ray studies showed a uniform increase in the translucency of his right lung while his left lung appeared relatively normal. Bronchospirometric studies showed that only 10 per cent of the total lung O_2 and CO_2 exchange was carried on by the right lung. This patient was considered to have unilateral obstructive emphysema. Patient 8 complained of dyspnea on exertion and chest x-ray examination showed one solitary cyst in each lung. Shortly after these studies, the cyst in one lung was removed and the diagnosis was verified. Patient 9 had a history of asthmatic attacks since early childhood, and his physical examination and chest x-rays were compatible with the diagnosis of bronchial asthma. Patient 10 had a history of pneumonia and empyema many years previously and a bronchopleurocutaneous fistula which persisted following open drainage of the chest. This fistula was obvious on physical examination and was occluded during pulmonary function tests. Patient 11 had a history of tuberculosis, and signs of fibro-cavernous tuberculosis in the right upper lobe by physical and x-ray examination. This patient had been referred from a sanatorium for lobectomy which was performed after these studies, the diagnosis being confirmed. Patient 12 had complained of cough 9 years previously, at which time his left lung was removed because of a squamous cell epithelioma of the bronchus. He now complained of dyspnea and the remaining lung was overdistended and considered to be emphysematous. Patients 14 and 15 were firemen who had been exposed to corrosive chemical fumes which severely damaged the upper respiratory tract, bronchi and bronchial tree, six weeks prior to these studies. Patient 15 still complained of shortness of breath, and granulation tissue in the trachea mucosa could be demonstrated by bronchoscopic examination at the time of these measurements. Patient 14 had been much less severely affected (as indicated by pulmonary function tests) and was symptom-free when studied. Both patients 16 and 17 had histories, physical examinations and x-ray findings compatible with acute diffuse interstitial fibrosis (37). This diagnosis was verified by a lung biopsy in patient 17. Patient 18 had the typical radiological features of pulmonary sarcoidosis, and biopsy of a cervical lymph node showed sarcoid changes. Patient 19 had generalized cutaneous scleroderma. His chest x-ray examination showed the extensive pulmonary fibrosis often seen with this disease, which was verified by pulmonary biopsy. Patient 20 complained of shortness of breath following an attack of varicella 4 months earlier. His heart was enlarged by x-ray examination, and pulmonary hypertension was demonstrated by right heart catheterization. Patient 21 also complained of shortness of breath, had a greatly increased pulmonary artery pressure by cardiac catheterization, and enlargement of the pulmonary outflow tract by x-ray examination. Since both of these patients had relatively normal ventilatory and distributory function and no evidence of an intracardiac shunt or other congenital heart defect was found by venous catherization in either, they were considered to have primarily pulmonary arterial disease. This was confirmed later at autopsy in patient 20. Patients 22 and 23 had histories, physical and x-ray findings suggestive of chronic rheumatic heart disease with mitral stenosis and well controlled congestive failure at the time of study. Patient 22 had hemosiderosis of the lungs by x-ray examination and increased left auricular pressure but no mitral stenosis when the valve was palpated at the time of operation. Biopsy of the auricular appendage was reported as fibroendocarditis. Patient 24 was asymptomatic, having had the absence, or obstruction of his right pulmonary artery discovered on a routine chest film and verified by pulmonary angiography. Since no alternate explanation was forthcoming, it was considered a congenital defect. Patient 25 had recently been diagnosed as polycythemia vera by blood measurements but at the time of the present study had been treated with radioactive phosphorus and had 5.4 million red cells per mm^3. He was otherwise in excellent health by history, physical and x-ray examination, and his ventilatory studies were in fact better than predicted for a normal subject. Patients 26, 27, and 28 all had the physical and x-ray findings of severe kyphoscoliosis; patient 26 had had his deformity since early childhood and in addition had tuberculosis of the hip at the time of study; patient 27 had been deformed since birth; patient 28 had been deformed since he had had rickets as an infant.

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THE PLASMA DISAPPEARANCE, EXCRETION, AND TISSUE DISTRIBUTION OF COBALT^{60} LABELLED VITAMIN B_{12} IN NORMAL SUBJECTS AND PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA

By AARON MILLER, HOWARD F. CORBUS, AND JOHN F. SULLIVAN

(From the Radioisotope and Medical Services, Boston Veterans Administration Hospital, and the Department of Medicine, Boston University School of Medicine, and Tufts University School of Medicine, Boston, Mass.)

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The serum concentration of vitamin B_{12} is increased in chronic myelogenous leukemia, and there is an associated increase in the *in vitro* binding of vitamin B_{12} by such sera (1, 2). On the other hand, the vitamin B_{12} concentration in the white cells and tissues in chronic myelogenous leukemia is not elevated (2). Mollin, Pitney, Baker, and Bradley (3) have demonstrated a delayed disappearance of cobalt^{58} labelled vitamin B_{12} from the plasma of patients with chronic myelogenous leukemia using an intravenous dose of 1.5 micrograms. It was the purpose of this study to measure plasma disappearance, tissue distribution, and excretion of a 4-microgram intravenous dose of cobalt^{60} labelled vitamin B_{12} in normal subjects and patients with chronic myelogenous leukemia. *In vitro* experiments would indicate that this dose exceeds the binding capacity of normal serum by about four fold, but is within the total binding capacity of chronic myelogenous leukemia serum (1, 2, 4).

Using a 4-microgram dose, a clear-cut differentiation could be made between normal controls and patients with chronic myelogenous leukemia.

METHODS

A. *Clinical material*

The subjects chosen for normal controls in this study were hospitalized patients convalescing from cerebrovascular accidents. Two to four months had elapsed since the onset of hemiparesis. All were asymptomatic save for residual paralysis, and none had evidence of anemia, renal disease, liver disease, congestive heart failure or infection. The diagnosis of chronic myelogenous leukemia was well established in eight patients. At the time of study, four patients had received no anti-leukemic therapy, and four had received some form of anti-leukemic therapy from three weeks to six months previously. Also studied were two patients with myeloid metaplasia, one patient with chronic lymphatic leukemia, one patient with Laënnec's cirrhosis with ascites and two postoperative patients with common bile duct drainage.

B. *Experimental plan*

1. *Plasma disappearance.* Four micrograms of cobalt^{60} labelled vitamin B_{12} was injected intravenously and its disappearance from the plasma determined by serum sampling and measurement.

2. *Tissue distribution.* Following injection, external monitoring of organ sites was carried out for periods up to 33 days. The concentration of radioactivity in the organs of two patients with leukemia was determined at post mortem. The concentration of radioactivity in red cells and white cells during the first twenty-four hours after injection was measured after separation from the plasma.

3. *Excretion.* Excretion of radioactivity in urine and stools was determined by measuring radioactivity in 24-hour urine and 7 to 10-day stool collections.

4. *Miscellaneous.* Two postoperative patients with T-tube drainage of the biliary tract were given cobalt^{60} labelled B_{12} intravenously and the radioactivity in 10-day bile collections determined. One patient with Laënnec's cirrhosis with massive ascites underwent paracentesis over a 2-hour period following the injection of cobalt^{60} labelled vitamin B_{12} and the radioactivity in the ascitic fluid was determined.

C. *Procedures*

1. *Plasma disappearance.* For intravenous injection, a suitable dilution of cobalt^{60} labelled vitamin B_{12} was made so that 5 ml. contained 4 micrograms of vitamin B_{12} and 3.0 microcuries of radioactivity. This was injected from a calibrated syringe into an antecubital vein. Ten ml. of blood was withdrawn with added heparin from the opposite arm at frequent intervals during the first two hours, and at 1 to 3 days thereafter for the duration of the study (up to 33 days). The percentage of the administered dose which remained in the plasma at any given time was determined by the formula:

$$\frac{\text{Counts per min./ml. plasma} \times \text{plasma vol. (ml.)}}{\text{Total counts per min. injected}} \times 100$$

2. *Radioactivity measurements.*

Scintillation well counting: Three-ml. plasma samples were counted in a thallium-activated sodium iodide well-

type scintillation counter. White cells were separated by the method of Buckley, Powell, and Gibson (5), and made up to 3-ml. volume and counted in the same manner. Washed red cells were also counted in 3-ml. volume. A standard was prepared by making a 1:500 dilution of the original injected sample, and duplicate 3-ml. aliquots were counted. Total injected radioactivity was calculated from the standard. All plasma samples collected during the first twenty-four hours after injection were counted with a counting error of less than 3 per cent. Samples collected after twenty-four hours were counted with a counting error of less than 5 per cent. Red cells and white cells were counted for 10 minutes to demonstrate negligible radioactivity.

Geiger-Müller well counting: Seventy-five-ml. aliquots of urine, bile, or concentrated ascitic fluid, and 75-gram aliquots of stool homogenate or organs were counted in a Texas-Allyn well-type GM counter. Seventy-five ml. of a 1:500 dilution of the injected material was used as a standard. Urine from normal patients and organ samples were counted with a counting error of less than 5 per cent. All other samples contained very little radioactivity, and were counted for 10 minutes.

External monitoring: External monitoring was done with a solid one-inch thallium-activated sodium iodide probe counter. A cobalt[60] standard was counted each morning to correct for daily variations in the counter. Counts were taken over liver, spleen, precordium, and thigh. Several areas over the liver and spleen were counted, and the area giving the highest counting rate was used.

3. *Serum vitamin B_{12}.* The concentration of vitamin B_{12} was determined by the Euglena gracilis method of Ross (6) as modified by Lear, Harris, Castle, and Fleming (7). The normal range reported by the latter group is 292 to 856 micromicrograms per ml.

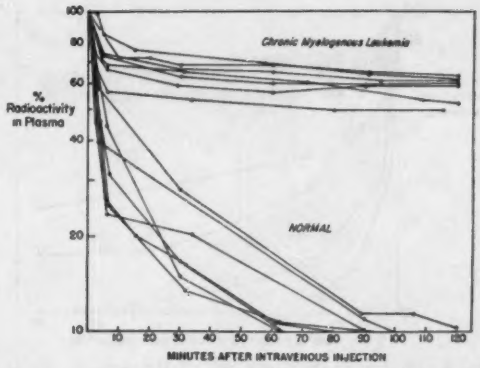

FIG. 1. DISAPPEARANCE OF COBALT[60] VITAMIN B_{12} FROM THE PLASMA OF NORMAL SUBJECTS AND PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA

4. *Plasma volume.* Plasma volume was determined by the Evans blue technique (8) in half of the patients, and estimated from the body weight in the remainder of the subjects using the formula:

$$\frac{\text{Plasmatocrit}}{100} \times 69 \text{ ml.} \times \text{body weight (kg.)}$$

RESULTS

A. *The disappearance of cobalt[60] labelled vitamin B_{12} from the plasma*

1. *Normal subjects.* The plasma radioactivity declined rapidly after injection; 35 to 55 per cent of the injected dose remained at the end of 5 min-

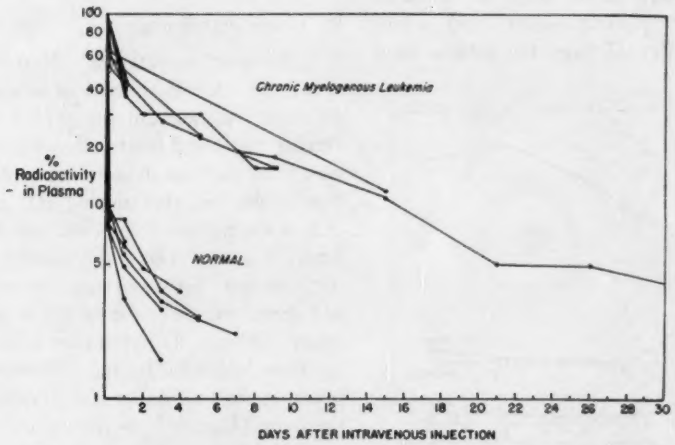

FIG. 2. DISAPPEARANCE OF COBALT[60] VITAMIN B_{12} FROM THE PLASMA OF NORMAL SUBJECTS AND PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA

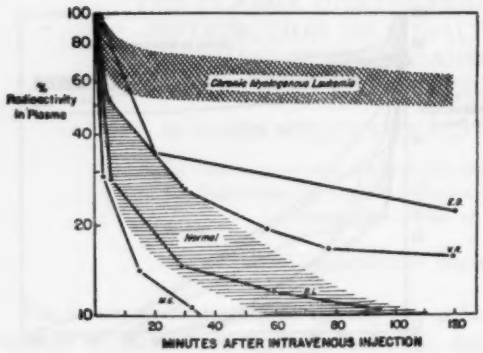

FIG. 3. DISAPPEARANCE OF COBALT^{60} VITAMIN B_{12} FROM THE PLASMA OF PATIENTS WITH VARIOUS DISORDERS

E. D. and V. R.—myeloid metaplasia.
M. G.—chronic lymphocytic leukemia.
B. L.—chronic myelogenous leukemia, treated and in remission.

utes and 8 to 10 per cent at two hours (Figure 1). Thereafter, the disappearance rate was slower (Figure 2). Because of the small amount of radioactivity present in the plasma after 24 hours, it was not possible to determine the slope of the disappearance curve accurately.

2. *Chronic myelogenous leukemia.* In seven out of eight patients with chronic myelogenous leukemia, plasma radioactivity disappeared more slowly: 50 to 63 per cent of the dose remained at two hours, and 38 to 42 per cent at 24 hours. After 24 hours, sufficient radioactivity remained in the plasma to determine the slope of the curve. This was approximately exponential, with a half time of 5 days. After 33 days, the plasma from one patient still contained 4 per cent of the administered dose. All seven of these patients showed evidence of activity of their disease (anemia, fever, splenomegaly), but not necessarily a high white count. Patient B. L., who was in complete clinical and hematological remission, showed a normal plasma disappearance (Figure 3).

3. *Other.* One patient with chronic lymphocytic leukemia showed normal plasma disappearance (Figure 3). Both patients with myeloid metaplasia showed a plasma disappearance which was intermediate between that of the normal subjects and the patients with chronic myelogenous leukemia (Figure 3).

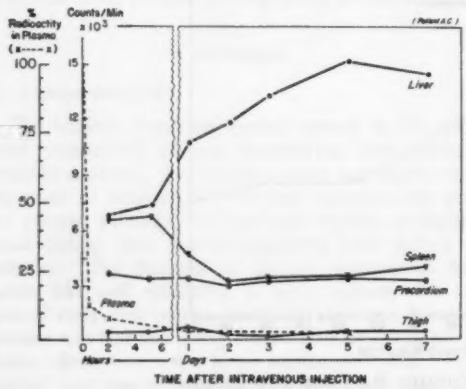

FIG. 4. DISTRIBUTION OF RADIOACTIVITY AFTER INTRAVENOUS COBALT^{60} VITAMIN B_{12} IN A NORMAL SUBJECT

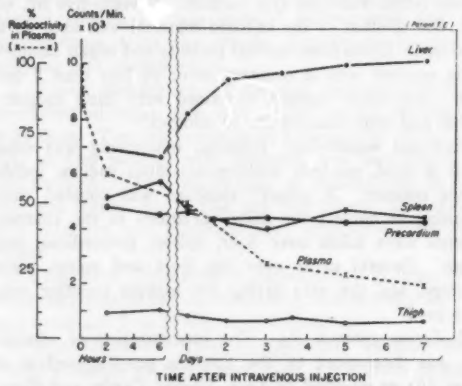

FIG. 5. DISTRIBUTION OF RADIOACTIVITY AFTER INTRAVENOUS COBALT^{60} VITAMIN B_{12} IN A PATIENT WITH CHRONIC MYELOGENOUS LEUKEMIA

B. *Tissue distribution*

1. *External monitoring.* External monitoring of normal subjects showed an increase in liver radioactivity throughout the period of observation. During the first 5 hours after injection, there was very little increase in counting rate over the liver, despite the fact that during this interval 90 per cent of the administered dose had left the plasma. From 5 to 48 hours, the counts over the precordium were constant, counts over the liver rose, and there was a consistent *fall* in counts over the spleen (Figure 4). Patients with chronic myelogenous leukemia, having a slower plasma disappearance, had a smaller rise in counting rate over the liver (Figure 5). No increase in concentration of radioactivity over enlarged spleens was found. Counting rates over organ sites in the patient with chronic lymphocytic leukemia with

normal plasma disappearance were the same as in the normal subjects.

2. *Red and white cells.* Red and white cells from both normal subjects and patients with leukemia contained no radioactivity.

3. *Post mortem.* The radioactivity in the organs of two patients with chronic myelogenous leukemia were determined at post mortem. Patient T. L. was injected 9 days and patient E. M. 23 days before death. The liver contained 39 to 42 per cent of the administered dose, the spleen 8 to 11 per cent and the other viscera less than one per cent (Table II). The concentration of radioactivity in the liver (counts per gram) was five to seven times greater than in spleen and other viscera.

C. *Excretion*

In the twenty-four hours after injection, normal subjects excreted 1 to 4 per cent of the administered dose in the urine, and chronic myelogenous leukemia patients, 0 to 2 per cent (Table I). Seven to ten-day stool collections were made in two normal subjects and contained no radioactivity. Ten-day bile collections contained less than 2 per cent of the administered dose. Ascitic fluid from a patient with cirrhosis (E. M.) was collected at intervals up to two hours during the measurement

TABLE I

Summary of clinical and laboratory data

Patient	Age	Serum vit. B_{12} concentration *μμg./ml.*	Wbc *per cu. mm.*	Plasma vol. *ml.*	Per cent radioactivity in plasma at 2 hrs.	Per cent radioactivity in plasma at 24 hrs.	24-Hour urinary excretion %
Normals							
1) N. G.	54		8,300	3,300	8	6	2
2) A. C.	65		5,800	3,000*	9	5	3
3) J. S.	63	530	8,500	3,200*	10	7	4
4) T. D.	64		5,000	3,000*	8	5	4
5) N. G.	55	364	9,600	3,400	8	3	4
6) V. K.	65	504	8,700	2,700*	10	6	1
7) H. M.	59		10,300	3,400*	8	4	2
Range					8–10	3–7	1–4
Mean					9	5	3
Chronic myelogenous leukemia							
1) T. L.	62		363,000	3,800	62	38	0
2) J. P.	57		91,100	3,600	63		
3) E. M.	32	9,266	12,600	3,800*	59		0
4) M. O.	53		118,000	3,300	53	42	
5) T. E.	62	2,046	313,000	3,900*	60	43	1
6) E. C.	52	7,020	71,000	4,000*	50		
7) W. S.	29	4,874	102,000	3,300*	59	42	2
Range					50–63	38–42	0–2
Mean					58	41	1
Miscellaneous							
Chr. myel. leukemia (In remission)							
1) B. L.	55		8,900	3,200	10		
Chr. lymphatic leukemia							
1) M. G.	62		160,000	3,200	7	3	2
Myeloid metaplasia							
1) E. D.	64	704	102,000	3,900	22		
2) V. R.	49		81,000	4,000	16	8	
Laënnec's cirrhosis							
1) E. M.	53	2,104	11,100	3,200*	10	3	2

* Plasma volume was determined by the Evans blue technique.

TABLE II

The tissue distribution of radioactivity in two autopsied patients with chronic myelogenous leukemia after intravenous Co^{60} vitamin B_{12}

Organ	Per cent injected radioactivity in total organ		Counts per sec. 100 gm. (wet weight)	
	T. L.	E. M.	T. L.	E. M.
Liver	42	39	54.9	63.1
Spleen	11	8	12.5	10.4
Kidney	1	1	14.3	11.3
Lung	1	1	10.3	7.0
Stomach	1		15.8	
Pancreas	1		7.3	

of the plasma disappearance. The entire 11 liters of fluid contained no radioactivity despite the fact that 90 per cent of the cobalt[60] labelled vitamin B_{12} had disappeared from the plasma within the two-hour interval.

D. *Serum vitamin B_{12} concentration*

In three normal subjects, the concentration of vitamin B_{12} in the serum ranged from 364 to 530 micromicrograms per ml. Serum vitamin B_{12} levels were elevated in four patients with chronic myelogenous leukemia ranging from 2046 to 9266 micromicrograms per ml. No correlation was found between the leukocyte count and the vitamin B_{12} level. Thus, patient E. M., with a normal white cell count, had the highest serum concentration of vitamin B_{12}. The serum concentration of vitamin B_{12} was 704 micromicrograms per ml. in E. D., a patient with myeloid metaplasia, and 2104 in E. M., a patient with cirrhosis.

DISCUSSION

The 4-microgram dose used in this study exceeds the total plasma vitamin B_{12} in the normal by about three fold. Because of the specific activity of the cobalt[60] labelled vitamin B_{12} available, the administration of a dose which would not significantly elevate the plasma concentration, *i.e.*, a tracer amount, would result in plasma counting rates not measurable by current techniques. Although the 4-microgram dose approaches a tracer amount in some of the chronic myelogenous leukemic patients, in the normal subjects the results may not be truly physiological because of the dosage employed.

In the normal subjects, intravenous vitamin B_{12} in the dosage used disappeared rapidly from the plasma in spite of minimal excretion in urine, stool, and bile. This decline in plasma concentration of cobalt[60] labelled vitamin B_{12} during the first two hours after injection is too rapid to be explained solely by mixing in extracellular fluid. Also, no radioactivity was recovered in ascitic fluid, although the patient with impaired liver function may not be completely comparable. The liver is thought to be the chief storage site of vitamin B_{12} (9, 10) and one might expect that the material would be taken up by the liver as it leaves the plasma. However, we found no concentration of radioactivity over this or any other organ site during the first five hours as measured by external monitoring. During this same period, direct sampling of erythrocytes and leukocytes revealed no measurable radioactivity. It is apparent that during the first five hours after injection, when virtually all of the material had left the plasma, no site of localization of the injected material was found with the techniques employed. A diffuse cellular uptake during this period could account for these observations.

However, external monitoring data indicate that the liver concentrates cobalt[60] labelled vitamin B_{12} and/or related compounds containing cobalt[60] beginning five hours after injection and continuing throughout the period of observation. This increase would seem too large to be accounted for by the small amount of material remaining in the plasma. Furthermore, during this period (five hours to two days) counting rates over the spleen continued to fall at a time when plasma and precordium counts were stable, suggesting cellular release of the vitamin or a cobalt-containing intermediate. Continued accumulation of the radioactivity in the liver was not due to an enterohepatic circulation, since negligible radioactivity was recovered in the bile.

The slow disappearance of vitamin B_{12} from the plasma of patients with chronic myelogenous leukemia probably reflects increased binding of this vitamin by the plasma (1, 2). This increased binding capacity correlated with the clinical state of the disease. Since serum concentrations decrease towards normal following successful therapy (1, 2), it is likely that this plasma abnormality also may disappear during remission (patient B. L.). Increased binding capacity did not correlate with the level of the white count, consistent with

the reports that increases in serum concentration of B_{12} do not correlate with the level of the white count (1, 2). The plasma disappearance curve can be used to estimate the daily plasma turnover of vitamin B_{12} in the leukemic patient. This is based on the assumption that the disappearance curve beginning at 24 hours, with a half time of 5 days, represents the physiological turnover of plasma vitamin B_{12}. This is likely, since this part of the curve is exponential. Also, the added radio-vitamin elevated the original serum concentration of vitamin B_{12} by less than 20 per cent, 24 hours after injection. Using this half time of five days, and knowing the original serum concentration of B_{12} and the plasma volume, the daily turnover rates in four leukemic patients were calculated, and ranged from 1.1 to 4.8 micrograms per 24 hours. This cannot be related to the normal, since the small amount of radioactivity in the normal plasma precludes calculation of daily turnover.

Although the serum from patients with chronic myelogenous leukemia binds increased amounts of vitamin B_{12}, the leukemic tissues from such patients do not appear to do so. No radioactivity was found in the leukocytes of leukemic patients at any time External monitoring over leukemic spleens revealed no concentration of radioactivity during the entire experiment (1 to 33 days), despite the disappearance of the radio-vitamin from the plasma. In two patients, the absence of splenic concentration of radioactivity as measured by external monitoring was corroborated by actual measurement of organ radioactivity at post mortem.

One patient with chronic lymphocytic leukemia was studied. Serum concentrations of vitamin B_{12} in this disease have been reported to be normal (1, 2). *In vivo* binding, as demonstrated by plasma disappearance, was normal in this patient. The serum concentration of vitamin B_{12} in patients with myeloid metaplasia has been reported to be somewhat elevated, although not as high as in chronic myelogenous leukemia (2). Although both patients with myeloid metaplasia were found to have a delayed disappearance of cobalt^{60} labelled vitamin B_{12} from the plasma, patient V. R. closely approximated the normal. In this patient histochemical staining of the polymorphonuclear leukocytes showed a decrease in the alkaline phosphatase as found in chronic myelogenous leukemia (11). However, by clinical criteria, this patient did not appear to have leukemia. It seems likely that these biochemical abnormalities may exist in varying degrees in this group of patients. It is of interest that the other patient with myeloid metaplasia, E. D., with a more delayed plasma disappearance, had a normal serum vitamin B_{12} level. Consistent with other reports (7) patient E. M. with cirrhosis had a high serum vitamin B_{12} level. However, this patient had a normal plasma disappearance. There have been reports of elevated serum concentrations of vitamin B_{12} in acute leukemia (12); unfortunately no such patients were available for the present study.

Mollin, Pitney, Baker, and Bradley (3) have recently described delayed plasma disappearance of an intravenous dose of 1.5 micrograms of cobalt^{58} labelled vitamin B_{12} in two patients with chronic myelogenous leukemia. It is difficult to compare our results because of the difference in the doses used. It is of interest that they have taken the 6-minute plasma sample as representative of 100 per cent of the injected dose. Our studies using both 1.5 (13) and 4 micrograms showed that 6 minutes following injection, 45 to 65 per cent of the dose had left the plasma in normal subjects and 15 to 34 per cent in the leukemic patients. These factors may explain the greater differences between the two groups of patients found in the present study.

The mechanism of the increased binding of vitamin B_{12} in the serum of chronic myelogenous leukemic patients is not known. Vitamin B_{12} is bound to the alpha fraction of serum globulin in both normal subjects and leukemic patients (1, 4). Increases in the concentration of this protein have been reported in many diseases not accompanied by an elevation of serum vitamin B_{12} concentration (14–16). Material liberated by chronic myelogenous leukemic white cells will bind vitamin B_{12} *in vitro*, and it has been suggested that this is the *in vivo* mechanism (2). However, the vitamin can be bound to normal white blood cells (17) as well as to other protein fractions. Further work is needed to clarify this problem.

In any case, the present study demonstrates a biochemical *plasma* abnormality in a disease which chiefly affects white cells. Other biochemical changes which have been described in leukemia, such as alterations in the concentrations of alkaline phosphatase (11) and histamine (18), have

been intracellular. This relatively simple method of demonstrating increased *in vivo* binding of vitamin B_{12} may be useful clinically in differentiating myelogenous leukemia from leukemoid states.

SUMMARY AND CONCLUSIONS

1. Four micrograms of cobalt^{60} labelled vitamin B_{12} were injected intravenously into normal subjects and patients with chronic myelogenous leukemia, and its plasma disappearance, tissue distribution and excretion were determined.

2. A rapid decline in plasma radioactivity occurred in normal subjects, contrasted with a slow decline in patients with clinically active chronic myelogenous leukemia. Excretion in urine and stools was negligible.

3. External monitoring of normal subjects, showed an increase in liver radioactivity throughout the period of observation, which was not associated with a comparable fall in plasma radioactivity. No concentration of radioactivity was found in spleen or white cells of patients with leukemia.

4. The plasma of patients with active chronic myelogenous leukemia can bind increased amounts of vitamin B_{12} *in vivo*. This may aid in differentiating chronic myelogenous leukemia from leukemoid states.

5. At the dosage level used, an increased binding of cobalt^{60} labelled vitamin B_{12} by chronic myelogenous leukemic tissue was not found.

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THE INFLUENCE OF EXTRACELLULAR THYROXINE-BINDING PROTEIN UPON THE ACCUMULATION OF THYROXINE BY TISSUE SLICES [1, 2]

By NORBERT FREINKEL,[3] SIDNEY H. INGBAR, AND J. THOMAS DOWLING [4]
WITH THE TECHNICAL ASSISTANCE OF BARBARA R. FINE

(From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass., and the Howard Hughes Medical Institute, Miami, Florida)

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Within the blood stream, thyroxine is distributed among the plasma proteins according to their relative binding affinities for this material. Thus, circulating thyroxine is principally associated with a moiety intermediate in electrophoretic mobility at pH 8.6 between the α_1 and α_2 globulins (1–6). This "thyroxine-binding protein" of plasma (TBP)[5] has been localized in subfractions of plasma prepared by Method 6 of Cohn, and it has been shown that all subgroups of Fraction IV-4 (especially IV-6 and IV-9) contain TBP greatly in excess of the normal serum proportions (6).

In the present studies, the presence of TBP has been demonstrated in extravascular fluids, and Cohn Fractions (7–9) and tissue slices have been employed for *in vitro* models designed to assess the possible significance of TBP in the partition of thyroxine between cellular and extracellular phases.

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[2] Presented in part at the 1955 meeting of the Endocrine Society, June 2–4, 1955, Atlantic City, New Jersey.

[3] Part of this work was performed during the tenure of a Fellowship of the American Cancer Society, Inc., recommended by the Committee on Growth of the National Research Council, 1953–1955.

[4] Fellow in Cancer Research of the American Cancer Society, Inc.

[5] To date, TBP has not been isolated. Therefore, it cannot be said whether TBP represents a single protein or several proteins which exhibit similar physico-chemical properties in plasma protein mixtures. Furthermore, it remains to be demonstrated whether the affinities for thyroxine result from some specific configurational characteristic of the TBP or merely from a richer endowment with more widely distributed thyroxine-binding residues.

MATERIALS

Thyroxine. Chromatographically pure preparations of I^{131}-labelled L- and D-thyroxine were obtained from a commercial source [6] and employed within three days of their arrival in the laboratory. The initial specific activity of the D-thyroxine was 14 mc. per mg.; specific activities of L-preparations varied from 30 to 50 mc. per mg. In experiments involving both isomers, specific activities were adjusted to identical levels by diluting the labelled L-thyroxine with unlabelled L-thyroxine sodium.[7]

Extracellular fluids. Thoracic duct lymph was obtained from a patient with spontaneous, unexplained chylothorax and femoral lymph was sampled from a patient in whom a right femoral lymphocoele had arisen one week following ligation of the superficial femoral vein. Knee joint fluid was secured from a patient with rheumatoid arthritis and multiple specimens of ascitic fluid were obtained from subjects with hepatic cirrhosis. Samples were collected with local anesthesia and sterile precautions by direct needle aspiration. Sterile specimens of urine were collected from pediatric patients with lipoid nephrosis. Cell counts of all fluids were performed immediately. Fluids were then analyzed for specific gravity, total protein, albumin, protein-bound iodine and stored at $-20°$ C prior to zone electrophoresis.

Tissues. Suitable pieces of heart, liver, and kidney were obtained from sheep, lambs, calves, or beef which had been sacrificed by exsanguination in the local abattoir. Tissues were wrapped in cellophane bags, placed on cracked ice, and transported to the laboratory in Dewar flasks.

Stock protein solutions. Solutions of mercaptalbumin (7)[8] and of Cohn Fractions II–III, IV–6, and V (8, 9) were prepared by dissolving the lyophilized proteins in isotonic saline. These solutions and sera obtained from two fasting euthyroid subjects were adjusted to pH 7.4 and to the same concentration of total protein (4.0 ± 0.1 gm. per cent) and protein-bound iodine (PBI: 10 ± 0.3

[6] Abbott Laboratories, Oak Ridge, Tenn.

[7] L-thyroxine sodium was 96.2 per cent pure on a dry basis and was kindly supplied by Dr. A. E. Heming of Smith, Kline & French Laboratories, Philadelphia, Penna.

[8] Mercaptalbumin which had been dialyzed against cysteine to remove mercury was kindly provided by Dr. Margaret J. Hunter of the Department of Biophysical Chemistry of Harvard Medical School.

μg. per cent) by the addition of appropriate quantities of 0.3 N NaOH or HCl, 0.15 N NaCl, and powdered L-thyroxine sodium.[7] Values for total protein and PBI were verified by chemical analysis.

Thyroxine-binding moieties in these stock protein solutions were compared by the zone electrophoretic and saturation techniques which have been described previously (6). Thus, the relative abundance of primary (*i.e.*, TBP) and secondary (*i.e.*, albumin, etc.) thyroxine-binding components in the stock protein solutions could be graded as follows:

Stock solution	Primary carriers	Secondary carriers
Cohn Fraction IV–6	+++++	0
Serum	+++	++
Cohn Fraction V	±	+++
Mercaptalbumin	0	+++
Cohn Fraction II–III	0	±

Since freeze-thawing does not alter TBP activity (6), the stock solutions were stored at −20° C and aliquots were removed for the preparation of suspending media.

Suspending media. For *in vitro* studies with tissue slices, Krebs-Ringer-Phosphate (KRP) (10) buffered at pH 7.4 and modified to contain 8.3 mM glucose and 0.8 mM $CaCl_2$ per liter were employed. Fresh solutions of KRP, both with and without supplemental protein (KRP + Prot., KRP–O–Prot.), were prepared for each experiment. KRP + Prot. mixtures were obtained by substituting the appropriate stock protein solution (v.s.) for an equivalent volume of NaCl. Such use of stock protein solutions facilitated the preparation of multiple KRP + Prot. media of identical pH, total protein, and PBI. In the KRP–O–Prot., total organic iodine was adjusted to levels corresponding to those in the KRP + Prot. by the addition of powdered L-thyroxine sodium.[7]

The same two batches of stock protein solutions were used in the suspending media of all of the experiments reported below.

METHODS

Protocols of individual experiments will be outlined in the "Results" section. General experimental procedures were as follows:

Analytical techniques. Paper electrophoresis was performed with apparatus and techniques described elsewhere (6). Concentrations of total protein were determined, in duplicate, by a modified biuret method (11) standardized by Kjeldahl determination of nitrogen in reference solutions of human albumin. Concentrations of albumin were measured, in duplicate, by the colorimetric, dye-absorption method of Rutstein, Ingenito, and Reynolds (12). A micro-Kjeldahl method was employed for analysis of total nitrogen in duplicate aliquots of tissue slices. Protein-bound iodine was estimated by the alkaline ash method of Barker, Humphrey, and Soley (13).[9]

Extracellular fluids. To undiluted 1-ml. specimens of lymph, nephrotic urine, knee joint fluid, and ascitic fluid, 0.06 μg. of L-thyroxine labelled with 1.8 μc. of I^{131} were added. Joint fluids were further supplemented with 75 units per ml. of hyaluronidase. The radioactive extracellular fluids were incubated for twenty minutes at room temperature; 0.020-ml. aliquots were then applied directly onto Whatman No. 3 filter paper for zone-electrophoresis at 110 volts. Studies were performed at pH 8.6 in a barbituric acid-sodium barbiturate buffer, r/2 0.5, and at pH 4.5 in an acetic acid-sodium acetate buffer, r/2 0.1.

Tissue incubation. Slices of heart, liver, and kidney cortex, 0.3 to 0.4 mm. in thickness, were prepared with a Stadie-Riggs microtome and placed in chilled 0.15 N NaCl. Prior to weighing, the slices were successively dipped into three portions of fresh saline in order to remove adherent blood. Tissues were quickly blotted, weighed on a torsion balance, and introduced into 2.7 ml. of suspending medium contained within the main compartment of chilled Warburg flasks. Within any single experiment, the tissue content of individual vessels did not differ by more than ± 10 mg. Representative slices were saved for nitrogen analyses. Three-tenths ml. of saline solutions containing 10 to 30 μc. of I^{131}-thyroxine per ml. were measured into the side-arms of the Warburg flasks. Preliminarily, these solutions had been adjusted to the same pH, and, with unlabelled thyroxine, to the same content of organic iodine as the KRP within the main compartments. The use of non-protein media within the side-arms of all of the flasks was designed to standardize the adsorption of radioactivity to glassware (6) during the preparative manipulations.

After the addition of 0.2 ml. of 10 per cent potassium hydroxide to the center well, flasks were placed in a thermostatically controlled water bath at 38° C and shaken at 112 cycles per minute. Gas spaces were flushed for five minutes with 100 per cent oxygen. Side-arms were emptied into the main compartments at timed intervals following fifteen minutes of temperature equilibration. Manometric techniques were as described elsewhere (14). Oxygen consumption (QO_2) was derived as cu. mm. O_2 per hour per mg. of initial wet weight of tissue.

Where more than nineteen flasks were required for a single experiment, tissues were incubated in 30-ml. beakers in the Dubnoff Shaking Incubator (shaking rate: 104 per minute; gas phase: 100 per cent oxygen; temperature: 38° C), and 0.3-ml. aliquots of radiothyroxine-saline solutions were directly introduced into the beakers following temperature equilibration.

Flasks were removed one hour after the "tipping-in" of radioactivity. Slices were blotted on filter paper, weighed on a torsion balance, and digested in 2 N NaOH. Suspending solutions were centrifuged immediately and residual radioactivity was determined from 1.0-ml. aliquots. In the *standard experimental procedure* no attempt was made to characterize the final radioactivity within the digested slices or the suspending media.

To evaluate the significance of thyroxine degradations during the one-hour *standard experimental procedure*, chromatographic studies were performed. Incubated slices were quickly frozen on chipped solid carbon dioxide, and then homogenized in 3.0 ml. of distilled water con-

[9] Iodine analyses were performed by Bio-Science Laboratories, Inc., 2231 Carmelina Avenue, Los Angeles 64, Calif.

taining 10^{-2} M propylthiouracil. One ml. of the homogenate was removed for assay of total radioactivity; the remainder was acidified to pH 3.0 with 10 N H_2SO_4 and extracted three times with 2-ml. portions of n-butanol. The butanol extracts were pooled; powdered propylthiouracil (3 to 5 mg.) was added, and the volume was reduced with a stream of nitrogen at room temperature. The residues were applied onto thin strips of Whatman No. 1 filter paper for forty-eight hours of descending one-dimensional chromatography at 20.0 + 0.5° C in isopentanol saturated with 6 N NH_4OH. This solvent system separates thyroxine from triiodothyronine, as well as from inorganic iodide and iodinated tyrosines (15). Developed strips were counted with a self-recording automatic strip-scanner and the percentage of the total radioactivity which migrated like unlabelled L-thyroxine carrier was determined. Measured aliquots of the suspending media were similarly treated for a) assay of total radioactivity, and for b) chromatographic fractionation of radioactive components.

In the *standard experimental procedure,* total recovery of radioactivity was derived as the sum of the counts remaining within the medium (counts per ml. medium × 3) plus the counts within the slices. Cellular "uptake" was expressed as a percentage of this total recovery (*i.e.,* tissue counts × 100/total recovered counts). In the chromatographic studies, total counts within the slices and media were corrected for radioactive components other than thyroxine in order to derive *corrected* cellular "uptakes."

Attempts to express "uptake" as a percentage of the theoretically added number of counts or as an absolute number of counts without regard to the radioactivity of the medium were complicated by variable adsorption of radiothyroxine (a) to the side-arms of the Warburg vessels prior to "tipping-in," especially at acidic pH; or (b) to the walls of the main compartment of the Warburg vessels following "tipping-in," especially into KRP–O–Prot. media. Since the precise contribution of glass surfaces to thyroxine distribution cannot be quantified, it was felt that "uptake," related to total recovery, best represented the partition of the residual radioactivity between a fluid extracellular phase and a solid tissue phase.

Cellular "uptake" was not corrected for adherent extracellular fluid. Arbitrary selection of a correction factor was not considered justified. Moreover, correction for extracellular fluid would not have altered relative intercomparisons and would have affected absolute values only slightly. For example, even if 200 mg. of tissues suspended in 3 ml. of fluid were contaminated with 40 per cent by weight of extracellular fluid, the maximum apparent "uptake" due to extracellular occlusions could not exceed 2.7 per cent of the total radioactivity [(0.200 × 0.40/3.0) × 100]. During true cellular accumulation, the suspending medium is depleted of radioactivity, and hence the potential occlusive error becomes even less. Nonetheless, it should be noted that the 2 to 3 per cent "uptakes" which were observed in some of the studies with KRP Fraction IV-6 may have represented predominantly extracellular radioactivity.

Radioactive assay. Electrophoretograms and chromatographic strips were scanned for radioactivity in an automatic strip scanner utilizing a gas-flow counter. The graphic recordings of radioactivity obtained from each strip were traced on hard paper of uniform thickness. The total curves and individual peaks were cut from the tracings and weighed, using a semi-automatic analytic balance of 0.05 mg. sensitivity. This permitted rapid and accurate integration of the total radioactivity and that of the individual peaks.

In slice experiments, aliquots of the suspending media and of the digested or homogenized tissues were diluted in volumetric flasks. To minimize adsorption to glassware, all dilutions were immediately made alkaline to phenolphthalein and supplemented with small amounts of human serum albumin and unlabelled thyroxine. Planchets were prepared and counted with a mica end-window Geiger-Muller tube connected to an automatic, self-recording sample changer. Geometry and self-adsorption characteristics were kept constant.

Other liquid samples were counted directly in well-type scintillation counters containing crystal detector heads of thallium-activated sodium iodide.

In all radioactive analyses, sufficient counts were observed to reduce the probable error of the measurement to less than ± 3 per cent.

RESULTS

Thyroxine-binding components in extracellular fluids

All fluids contained a thyroxine-binding component which simulated the zone-electrophoretic characteristics of the TBP of plasma (1–6, 14, 16) (Figure 1). Thus, part of the added radiothyroxine migrated intermediate between the α and the α_2 globulins at pH 8.6 and towards the anode at pH 4.5. In all extracellular fluids, albumin constituted the chief secondary carrier for thyroxine.

Unbound thyroxine does not display comparable mobilities under these conditions (6). Significant contamination of extracellular fluids with blood may be excluded on the basis of the low red blood cell counts (Figure 1).

The interaction of thyroxine with tissue slices

During incubation *in vitro,* surviving slices accumulated radiothyroxine from the suspending media. Experiments were performed with slices of lamb, sheep, calf and beef kidney cortex, heart, and liver. In all studies, radioactive "uptakes" were greatest in KRP–O–Prot. and varyingly reduced in KRP + Prot. At equivalent concentrations of the total protein, the KRP + Prot. mix-

tures impaired cellular "uptake" in direct proportion to their content of specific thyroxine-binding moieties. Thus, at protein concentrations ranging from 0.5 to 2.0 gm. per cent, the inhibitory potentials of the KRP + Prot. mixtures could be graded as follows: KRP + Fract. IV-6 > KRP + serum > KRP + Fract. V or mercaptalbumin > KRP + Fract. II–III.[10] Table I represents one such experiment wherein all vessels were incubated in triplicate in order to assess the reproducibility of "uptakes" in the *standard experimental procedure*.

Chromatographic fractionation of sheep liver, kidney, and heart slices and KRP + Prot. and KRP–O–Prot. media following one hour of incubation revealed that unaltered radiothyroxine constituted more than 80 per cent of the total radio-

[10] The differences between KRP–O–Prot. and KRP + Fract. II–III may be more apparent than real. The absolute cellular accumulations of radioactivity (*i.e.*, counts per gm.) did not differ markedly in these two media. However, total recovery of radioactivity was consistently greater in KRP + Fract. II–III. Therefore, "uptake," when expressed as a percentage of the total recovery, was less in KRP + Fract. II–III than in KRP–O–Prot. It is possible that the inclusion of Fract. II–III in the suspending medium may have only impaired the adsorption of radioactivity to glassware without altering absolute cellular accumulation. On the other hand, all of the other KRP + Prot. media effected a reduction of the absolute as well as the fractional "uptake" of radiothyroxine from the extracellular phase.

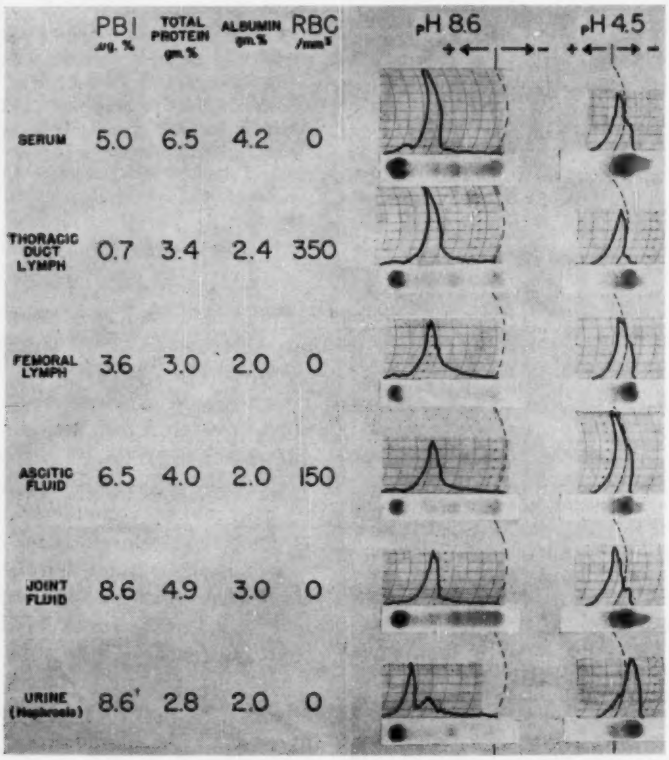
PBI μg. %
TOTAL PROTEIN gm. %
ALBUMIN gm. %
RBC /mm³
pH 8.6
pH 4.5
SERUM 5.0 6.5 4.2 0
THORACIC DUCT LYMPH 0.7 3.4 2.4 350
FEMORAL LYMPH 3.6 3.0 2.0 0
ASCITIC FLUID 6.5 4.0 2.0 150
JOINT FLUID 8.6 4.9 3.0 0
URINE (Nephrosis) 8.6† 2.8 2.0 0

FIG. 1. THYROXINE-BINDING BY EXTRACELLULAR FLUIDS

The darkened lines represent the scanning pattern of the distribution of radioactivity following paper electrophoresis at pH 8.6 and at pH 4.5. Electrophoretograms stained with bromphenol blue have been reproduced underneath the scanning patterns. However, staining detail has been lost in the photographic reproduction. Values for protein-bound iodine (PBI) represent endogenous levels prior to supplementation with I^{131}-labelled L-thyroxine. † Total iodine in the nephrosis urine: 15.4 μg. per cent.

TABLE I

"Uptake" of L-radiothyroxine by surviving slices of lamb kidney cortex: Reproducibility of "uptake" measurements *

Suspending medium +	"Uptake" (% of total recovery)	QO_2†
O-Prot.	34.6, 33.4, 35.6	1.19
Fract. II–III	20.2, 24.4, 23.0	1.25
Mercaptalb.	13.0, 12.6, 11.8	1.22
Serum	8.2, 8.2, 7.0	1.38
Fract. IV–6	3.4, 3.4, 3.2	1.42

* Tissues incubated for one hour in triplicate; total protein in KRP + Prot. media ranged from 1.5 to 1.6 gm. per cent; nitrogen content of tissue slices averaged 4.31 mg.

† QO_2 is derived as cu. mm. O_2 per hour per mg. of initial wet weight and represents average of triplicate analyses.

activity within all systems. Radioactive breakdown products partitioned between slices and media in such a fashion so that "uptakes" which had been corrected for radioactive components other than thyroxine did not differ significantly from uncorrected "uptakes" based on the total recovered radioactivity (Table II). The finding that the breakdown of thyroxine is minimal during one hour of incubation *in vitro* is in accord with the observations of Albright, Larson, and Tust in the rat (17) and supports the use of uncorrected "uptakes" in the *standard experimental procedure.*

To evaluate the kinetics of cellular accumulation, tissues were incubated in the Dubnoff apparatus and multiple analyses were secured at timed intervals. "Uptake" was not instantaneous, but rather slowly approached ultimate limiting values in all media. At all times throughout the equilibration period, cellular "uptakes" were proportional to the thyroxine-binding components in the suspending media (Figure 2). Although the potential magnitude of thyroxine degradation during prolonged incubation has not been assessed, it should be noted that the qualitative interrelationships after 360 minutes of incubation did not differ from the findings at 30 minutes.

Characteristics of the cellular/extracellular distribution of thyroxine in vitro

Reversibility: Tissues were suspended in 3 ml. of KRP–O–Prot. containing I^{131}-labelled L-thyroxine. Following one hour of incubation, control flasks were removed. To the remaining flasks were added 1 ml. of either KRP–O–Prot. or KRP + Fract. IV-6 (5.9 gm. per cent). Paired groups of flasks were removed at 20-minute intervals during the next hour. Analysis revealed that the introduction of KRP + Prot. was followed by a rapid removal of radioactivity from the slice (Figure 3). The tissue content of I^{131} approached a low, apparently limiting value at 40 minutes. Where only KRP–O–Prot. had been added, thyroxine distribution was not significantly altered.

TABLE II

"Uptake" of L-radiothyroxine by surviving slices of lamb tissues: Corrected vs. standard "uptake" *

Tissue	Suspending medium +	"Uptake" Standard†	"Uptake" Corrected‡
Kidney cortex	O-Prot.	49.3	48.6
	Fract. II–III	26.6	24.8
	Fract. IV–6	3.9	3.7
Liver	O-Prot.	49.8	48.2
	Fract. II–III	32.1	30.1
	Fract. IV–6	5.1	4.4
Heart	O-Prot.	74.3	80.0
	Fract. II–III	32.8	34.8
	Fract. IV–6	4.9	4.5

* Tissues incubated for one hour; total protein in KRP + Prot. media ranged from 1.4 to 1.5 gm. per cent.

† Standard "Uptake": Tissue I^{131} expressed as a percentage of total recovered radioactivity.

‡ Corrected "Uptake": Tissue radioactivity expressed as a percentage of chromatographically-identified radiothyroxine within slices and media.

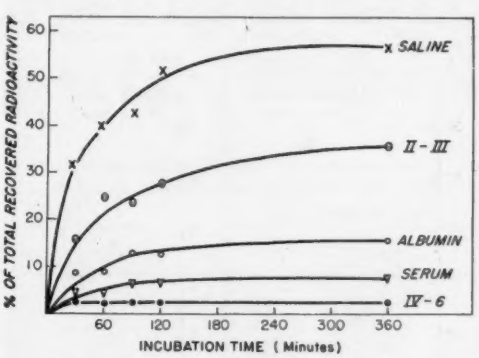

FIG. 2. "UPTAKE" OF L-RADIOTHYROXINE BY SURVIVING SLICES OF CALF HEART

"Saline" refers to KRP–O–Prot. and the "II–III, albumin, serum and IV–6" refer to the various KRP + Prot. suspending media. Cellular "uptake" is expressed as a percentage of the total recovered radioactivity. Nitrogen content of the incubated slices ranged from 3.42 to 3.76 mg; total protein in the KRP + Prot. mixtures varied from 1.4 to 1.5 gram per cent.

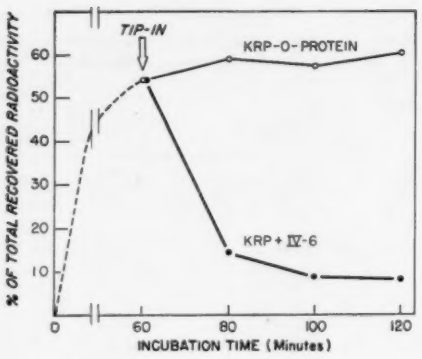

FIG. 3. REVERSIBILITY OF THE TISSUE "UPTAKE" OF L-RADIOTHYROXINE

Slices of sheep heart were incubated for one hour in KRP–O–Prot. containing I^{131}-labelled L-thyroxine. Control flasks were removed and 1-ml. portions of non-radioactive KRP–O–Prot. or KRP + Fract. IV–6 were added to the remaining flasks.

Protein concentration: When the concentration of Fraction IV-6 in the suspending medium was kept constant and slice weight was varied, cellular "uptake" varied in relation to tissue mass. Conversely, when slice weight was kept constant, and IV-6 was varied, cellular "uptake" was inversely proportional to the extracellular TBP (Figure 4).

Similar relationships have been observed with simple plasma protein mixture (6). To document the parallelisms, paired experiments were performed wherein 1) varying amounts of Fraction IV-6 were incubated with constant amounts of surviving tissue slices and the distribution of radiothyroxine was quantified by direct analysis, and 2) varying amounts of IV-6 were incubated with constant amounts of mercaptalbumin and the distribution of radiothyroxine was assessed by paper electrophoresis. In both systems, the partition of thyroxine was conditioned by the availability of TBP (Figure 5).

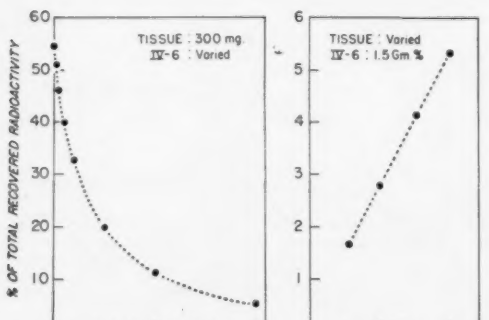
TISSUE : 300 mg.
IV-6 : Varied
TISSUE : Varied
IV-6 : 1.5 Gm %
% OF TOTAL RECOVERED RADIOACTIVITY
FRACTION IV-6 (Gm.%)
KIDNEY SLICES (Mg. Wet Wt.)

FIG. 4. "UPTAKE" OF L-RADIOTHYROXINE BY SURVIVING SLICES OF LAMB KIDNEY CORTEX

The effects of variations in tissue mass and in the concentrations of extracellular KRP + Fract. IV–6 are demonstrated. Nitrogen content of the kidney slices averaged 1.72 mg. N per 100 mg. of initial wet weight.

Thyroxine concentration: L-thyroxine within all suspending media was increased by the addition of unlabelled thyroxine. Concentrations of cellular and extracellular proteins were kept constant. This resulted in an augmented percentile "uptake"

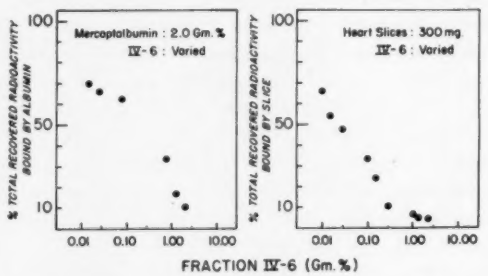
Mercaptalbumin : 2.0 Gm. %
IV-6 : Varied
Heart Slices : 300 mg.
IV-6 : Varied
% TOTAL RECOVERED RADIOACTIVITY BOUND BY ALBUMIN
% TOTAL RECOVERED RADIOACTIVITY BOUND BY SLICE
FRACTION IV-6 (Gm. %)

FIG. 5. SIMILARITIES IN THE DISTRIBUTION OF THYROXINE IN MIXTURES OF PLASMA PROTEINS AND IN CELLULAR SYSTEMS

All solutions were adjusted with L-thyroxine to an initial concentration of 0.15 μg. of organic iodine per ml. Heart slices were obtained from lambs and averaged 1.68 mg. of nitrogen per 100 mg. of initial wet weight.

and in a greater absolute accumulation of thyroxine than might be anticipated on the basis of a fixed cellular/extracellular distribution equilibrium (Table III). Systems containing KRP + Fract. IV-6 were least affected by the added thyroxine. Thus, in the experiment depicted in Table III, the change in cellular "uptake" in KRP + IV-6 is of questionable significance although the increases in all other media exceed the limits of methodological variability. Enhancement of "uptake" was most pronounced at low concentrations of extracellular proteins. Completely protein-free media were not achieved. Protein concentrations of 20 to 30 mg. per cent (with slices of kidney and heart) or 100 to 150 mg. per cent (with slices of liver) were frequently demonstrated in suspending media following incubation in KRP–O–Prot. Whether

TABLE III

"Uptake" of L-radiothyroxine by surviving slices of lamb kidney cortex: Relationship to thyroxine concentration *

Suspending medium +	"Uptake" (% of total recovery) Low Tx.†	High Tx.‡
O-Prot.	53.9	68.1
Fract. II–III	26.1	45.9
Fract. V	18.4	25.8
Serum	8.1	16.3
Fract. IV–6	4.1	4.8

* Tissues incubated for one hour; total protein in KRP + Prot. media was 0.5 gm. per cent; nitrogen content of tissue slices averaged 4.40 mg.

† Low Tx.: Chemically-estimated protein-bound iodine in KRP + Prot. media ranged from 2.9 to 3.4 μg. per cent.

‡ High Tx.: Chemically-estimated protein-bound iodine in KRP + Prot. media ranged from 9.7 to 11.0 μg. per cent.

these proteins originated as adherent plasma proteins or were leached into the medium during incubation cannot be stated.

Thyroxine isomers: Differences in the metabolism of thyroxine isomers *in vivo* were studied in a euthyroid volunteer and in a patient with untreated primary myxedema. Intravenous doses of 70 μc. (6 μg.) of I^{131}-labelled D-thyroxine and 50 μc. (2 μg.) of I^{131}-labelled L-thyroxine were given to each subject at intervals of six weeks. Daily blood samples were secured for nine days following each administration. In both subjects, the early dilution (*cf.* blood level Day 1) and the subsequent rate of disappearance (*cf.* blood levels Day 2 to Day 9) of D-thyroxine exceeded that of the L-isomer (Figure 6). Rall, Robbins, Becker, and Rawson (18) have reported similar observations.

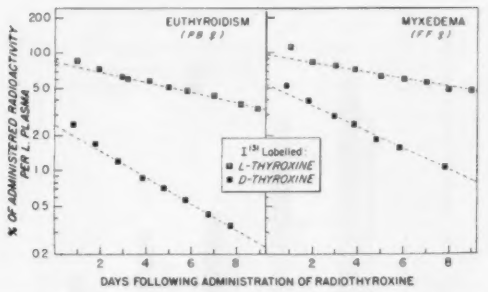

FIG. 6. THE DISAPPEARANCE OF LABELLED THYROXINE ISOMERS FROM THE CIRCULATION OF HUMAN SUBJECTS

For *in vitro* studies, tissue slices were incubated in "two isomer" and in "one isomer" systems. In the former, protein and non-protein KRP preparations were adjusted to an organic iodine content of 0.07 μg. per ml. with unlabelled L-thyroxine and then supplemented with 0.06 μg. per ml. of either D- or L-radiothyroxine. The isomeric mixtures were designed to simulate the conditions *in vivo* which obtain following the administration of D-thyroxine. In the "one isomer" systems, KRP preparations were supplemented exclusively with 0.14 μg. per ml. of either D- or L-radiothyroxine. Concentrations of proteins in KRP–Prot. were sufficiently low (5 mg. per ml.) to minimize the contributions of the endogenous protein-bound iodine. Both the "one isomer" (Table IV) and the "two isomer" (Figure 7) systems yielded

TABLE IV

"Uptake" of thyroxine isomers by surviving slices of lamb kidney cortex: Relationship to oxygen tension *

Suspending medium +	Initial pH	100% Oxygen Final pH	"Uptake"	QO_2	100% Nitrogen Final pH	"Uptake"	QO_2
		L-Thyroxine †					
O-Prot.	7.23	7.12	46.2	1.16	6.88	48.6	
Fract. II–III	7.24	7.28	26.1	1.20	6.91	35.4	
Serum	7.32	7.40	8.1	1.18	6.95	10.7	
KRP + IV-6	7.26	7.30	4.1	1.36	7.03	3.2	
		D-Thyroxine †					
O-Prot.	7.23	7.12	53.9	1.17	6.90	55.6	
Fract. II–III	7.24	7.30	35.4	1.19	6.91	42.5	
Serum	7.32	7.40	18.9	1.13	7.00	18.5	
KRP + IV-6	7.26	7.32	8.2	1.40	7.07	6.1	

* Tissues incubated for one hour; total protein in KRP + Prot. media was 0.5 gm. per cent, "Uptake" calculated as percentage of total recovered radioactivity.

† "One isomer systems" (see text): Media supplemented to contain 0.14 μg. per ml. of either D- or L-radiothyroxine.

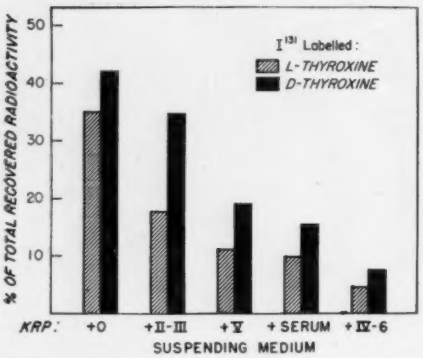

FIG. 7. "UPTAKE" OF THYROXINE ISOMERS BY SURVIVING SLICES OF LAMB KIDNEY CORTEX

All KRP media were adjusted with unlabelled L-thyroxine to contain 0.07 μg. of organic iodine per ml. "Two isomer systems" (see text) were prepared by supplementing the suspending solutions with 0.06 μg. per ml. of either D- or L-radiothyroxine. Concentrations of total proteins in the KRP + Prot. mixtures ranged from 1.9 to 2.0 gm. per cent.

similar results. The percentile "uptake" of the D-thyroxine varied inversely with the abundance of thyroxine-binding moieties in the suspending media. Cellular accumulation of D-radiothyroxine was greatest in KRP–O–Prot. and least in KRP + IV-6. The "uptake" of the D-material exceeded that of the L-isomer in all media. Differences in the "uptake" of the isomers were least marked in KRP–O–Prot.

pH: KRP–O–Prot. and KRP + Fract. IV-6 media were adjusted to varying pH levels prior to the introduction of tissue slices. In KRP + Fract. IV-6, cellular "uptake" varied inversely with pH (Figure 8). For kidney cortex and heart, pH dependencies were neither as marked nor as consistent in the KRP–O–Prot. systems.

In all suspending media, the total recovery of radioactivity was consistently diminished at low pH. This enhances the significance of the increased cellular "uptakes" from acidic KRP + Fract. IV-6, since the reverse might be anticipated on the basis of the relationship between "uptake" and thyroxine concentration which has been described above.

Metabolic relationships in vitro

The effect of cellular "uptake" upon cellular respiration: Cellular respiratory activity was unaltered by variations in cellular "uptake." The profound changes in "uptake" which were effected by substituting KRP–Prot. for KRP–O–Prot., or by increasing the extracellular concentration of thyroxine, did not significantly affect QO_2.

However, the presence of IV-6 in the suspending medium was often, although not invariably, associated with an immediate and a sustained fifteen to thirty per cent augmentation of cellular respiration. This was unrelated to the absolute amount of thyroxine within the system. In the experiment which is partly pictured in Figure 4, the QO_2 values of surviving kidney slices in KRP containing 40, 20, 15, 10, 2.5, 0.5, 0.1, or 0.0 mg. per ml. of Fraction IV-6 were 1.73, 1.50, 1.51, 1.38, 1.29, 1.32, 1.32 and 1.33, respectively. The concomitant respective "uptakes" were 3.0, 3.4, 4.2, 5.2, 20.0, 39.0, 51.1, and 54.5 per cent. Occasional immediate enhancement of respiration also occurred in media which had been supplemented with Fraction V or serum. These inconstant effects of certain proteins could not be correlated with any variant of the incubation procedure, nor could they be duplicated with triiodothyronine. The phenomenon remains unexplained and is currently under investigation.

The effect of cellular metabolism upon cellular "uptake": Substitution of 100 per cent nitrogen for 100 per cent oxygen in the *standard experimental procedure* did not produce consistent al-

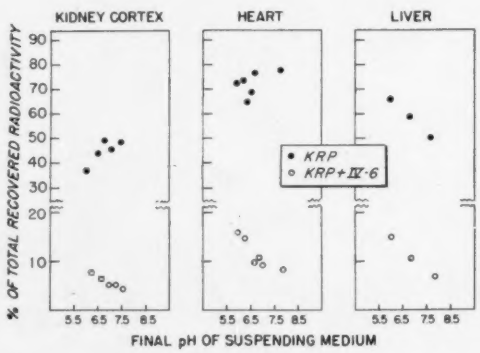

FIG. 8. THE EFFECT OF pH UPON THE "UPTAKE" OF L-RADIOTHYROXINE BY SURVIVING SLICES OF LAMB TISSUES

Final pH values above 7.5 were obtained by substituting Tris (hydroxymethyl) aminomethane (0.1 M) for phosphate buffer in the preparation of the most alkaline suspending media.

terations in the "uptake" of radiothyroxine by surviving slices (Table IV). When surviving slices were incubated for one hour at 8° C rather than at 38° C, "uptakes" were significantly depressed (Table V). "Uptakes" were not impaired by boiling duplicate sets of weighed slices for ten minutes prior to incubation (Table V). The "uptakes" by boiled tissues from KRP–O–Prot. and KRP + Fract. II–III media were even greater than those of unboiled controls. The enhancement of "uptake" may have resulted from loss, during boiling, of those cellular or interstitial thyroxine-binding components which are leached into the incubating medium during the *standard experimental procedure* (v.s.), and which ordinarily augment the thyroxine-binding capacity of the extracellular phase. However, alternative explanations cannot be excluded.

TABLE V

"Uptake" of l-radiothyroxine by slices of sheep kidney cortex: Relationship to temperature and tissue viability *

Suspending medium +	"Uptake" (% of total recovery)		
	Surviving slices		Boiled slices
	38° C	8° C	38° C
O-Prot.	51.8	19.0	81.7
Fract. II–III	12.7	7.7	33.3
Fract. IV–6	3.0	1.9	2.9

* Tissues incubated for one hour; total protein in KRP + Prot. media ranged from 1.8 to 1.9 gm. per cent.

DISCUSSION

Certain lines of evidence would suggest that the combination of thyroxine with plasma TBP may be of importance in the extravascular disposition of thyroxine. In an earlier report (19) it had been noted that the values for the turnover and distribution of radiothyroxine in humans (19, 20–23) are somewhat similar to those of such macromolecules as labelled plasma proteins (24–27) or highly associated complexes of dye and protein (28). Since that time, Alpers and Rall (29) have reported the presence of plasma TBP in cerebrospinal fluid, and, in the present communication, TBP has been demonstrated in other normal and abnormal extravascular fluids. These recent findings would suggest that plasma TBP, like other plasma proteins, is distributed in a portion of the body waters which includes freely-exchanging intra- and extravascular components. Moreover, the data lend support to the concept of a "thyroxine-space" whose anatomical boundaries may be delimited by the thyroxine-binding protein. It is quite likely that free, unbound thyroxine is involved in the ultimate transcellular exchange of thyroxine. Nonetheless, from the available evidence it may be inferred that extracellular thyroxine is chiefly in association with a plasma protein carrier, and that tissue thyroxine must be abstracted from such a reservoir.

In the present studies, *in vitro* models were designed to assess the static factors which may influence this partition of thyroxine between cellular and extracellular phases. A high degree of specificity was afforded by the availability of plasma protein fractions of known and varied thyroxine-binding capacities.[11] Furthermore, by chromatographic techniques it could be demonstrated that during the *standard experimental period* of one hour, cellular degradation of thyroxine was insufficient to influence appreciably the static distribution phenomena. Thus, simple systems could be devised to characterize a) the cellular "uptake" of thyroxine, b) the relationship between cellular "uptake" and extracellular events.

"Uptake" by tissues *in vitro* was shown to be dependent upon temperature but unrelated to cellular metabolic activity. Anoxia did not depress "uptake" nor did such drastic procedures as denaturation of tissue proteins by boiling. Thus, it is apparent that the establishment of concentration differentials for thyroxine between slices and suspending media constitutes an equilibrium binding phenomenon rather than an "active" transport.

[11] The use of human plasma fractions in association with animal tissues may be questioned. Obviously, systems containing homologous cellular and extracellular phases would have been preferable. This was possible for studies involving KRP + serum. In preliminary experiments, comparable modifications of slice "uptakes" were effected with KRP + Homologous Animal Serum and KRP + Heterologous Human Serum. For experiments with Cohn Fractions, homologous systems were not feasible. However, studies with animal sera (30) have demonstrated that in sheep, lambs, beef and calves, as in humans, circulating thyroxine is principally associated with a discrete alpha-globulin moiety. Moreover, albumin constitutes the chief secondary carrier for thyroxine in all these species. The gross parallelisms in plasma transport suggest that extravascular distribution phenomena may be similar also, and that heterologous systems may be employed for their qualitative evaluation.

Crispell and Coleman (31) have reached similar conclusions about the binding of radiothyroxine by intact human red cells (32). As such, it is not surprising that many of the present findings simulate previous observations which were made with mixtures of plasma proteins (6). At that time, it was concluded that thyroxine is distributed among plasma proteins according to a reversible binding equilibrium between a finite amount of TBP and relatively unlimited quantities of secondary carriers of lesser binding affinities. For simplicity, an inert role was assigned to the thyroxine molecule and the binding partition between the competing proteins was described in terms of a two-component system. Certainly much of the present data may be formulated in a similar fashion.

First, it has been demonstrated that the "uptake" of thyroxine by surviving slices progressively approaches limiting values. Admittedly, this could represent cellular saturation. However, since the ultimate "uptake" is a function of the protein content of the suspending medium, it seems more likely that the asymptote is determined by some form of distribution equilibrium between cellular and extracellular binding moieties. Secondly, the capacity of extracellular media to diminish the cellular "uptake" of thyroxine is directly proportional to their content of soluble thyroxine-binding proteins (especially TBP). This correlation would indicate that changes in "uptake" are not due merely to non-specific van der Waals' interactions between thyroxine and extracellular macromolecules. Thirdly, thyroxine already bound by the cell can be withdrawn therefrom by the introduction of TBP into the medium, much as the addition of TBP to plasma protein mixtures can recover thyroxine from albumin (6). Finally, as one would expect from equilibrium principles, the distribution of a constant amount of thyroxine can be predictably altered by varying the concentration of either the cellular or the extracellular binding reactants.

In mixtures of plasma proteins, thyroxine can be displaced from TBP onto albumin by the addition of increasing amounts of thyroxine (6). Similarly, cellular "uptake" can be augmented by increasing the concentration of extracellular thyroxine. The findings would indicate that the partition of thyroxine in both systems is conditioned not only by the relative abundance of binding moieties, but also by the absolute amount of thyroxine which is to be bound. For mixtures of plasma proteins, this thyroxine-dependent spectrum of distribution equilibria has been attributed to relative saturation of TBP. A similar explanation may be invoked in the slice preparation and it should be noted that truly protein-free suspending media were not achieved even in the KRP–O–Prot. systems. However, it cannot be excluded that some of the enhancement of cellular "uptake" may have resulted from saturation of adsorbing glass surfaces, or from some intrinsic characteristic of cellular binding reactions, which is independent of extracellular proteins.

In some of the other experiments, analogy becomes more complicated. For example, the uptake of D-thyroxine from all suspending media, exceeded that of the L-isomer. Robbins and Rall (33) noted little differences in the binding of thyroxine isomers by TBP. Contrariwise, Larson and Albright (34) reported that plasma TBP binds L-thyroxine much more tenaciously than the D form. In the face of these contradictory findings it remains to be elucidated whether the preferential tissue accumulation of D-thyroxine *in vitro* and the more rapid turnover of D-thyroxine *in vivo* are conditioned solely by extracellular factors.

Similarly, it should be noted that TBP has not yet been isolated, the cellular sites for binding thyroxine have not been defined, and even the isoelectric point for thyroxine has not been determined. Thus, more than descriptive analysis of the increase of cellular "uptake" which occurs in KRP + IV-6 media at acidic pH is not warranted. Since thyroxine is an amino acid, and plasma TBP and tissues are protein, electrostatic interactions (35) of all three are theoretically capable of independent and interdependent modifications by pH. pH may alter not only TBP or cellular binding affinities, but also the configurational characteristics of the thyroxine molecule which make for its binding at the same or at different sites by TBP or cells.

Limitations of the present data should be recognized. The site of cellular "uptake" has not been localized; thus, distinction cannot be made between simple binding onto cell surfaces and "uptake" which reflects penetration to subcellular particles beyond cell-membrane barriers. Moreover, the

possibility that extracellular proteins may facilitate *transcellular* flux has not been evaluated. Current investigations in this laboratory are focused in these directions.

For the moment, the precise correlations between the slice studies and the intact animal are conjectural. However, it does not seem unlikely that the *in vitro* system reproduces the static phenomena which obtain *in vivo*. Hence, in the whole animal, at any single instant, the partition of thyroxine between cellular and extracellular phases may be conditioned by the absolute concentration of thyroxine, and by the quantitative balance between cellular and extracellular binding moieties. Furthermore, on the basis of the slice experiments, it seems plausible that this partition may be acutely altered by such sequelae of intracellular metabolism as pH changes independent of fluctuations in the absolute concentration of any of the interacting variables.

In more dynamic terms, thyroxine exchanges in the living animal differ markedly from the one-hour slice experiments. *In vivo,* there is a continuous irreversible removal of thyroxine from the "thyroxine-space" through cellular degradation and fecal losses. This is counterbalanced by the delivery of an equivalent amount of new thyroxine from the intrathyroidal hormone stores. In addition, there is probably a constant renewal and degradation of the cellular and extracellular thyroxine-binding components. All of these factors must be considered in attempts to extend the available information into any formulation for the intact animal.

The sequence may be written:

$$\begin{aligned} &+ (\mathrm{TBC})_{EC} \rightleftharpoons (\mathrm{TBC})_{EC}\,\mathrm{Tx} \\ \mathrm{Tx} \\ &+ (\mathrm{TBC})_{C} \rightleftharpoons (\mathrm{TBC})_{C}\,\mathrm{Tx} \rightarrow \mathrm{T}' \end{aligned}$$

wherein:

Tx = Thyroxine

$(\mathrm{TBC})_{EC}$ = Extracellular thyroxine-binding components (principally plasma TBP)

$(\mathrm{TBC})_{C}$ = Cellular thyroxine-binding components (presumably protein in nature but as yet unidentified)

T′ = Products of cellular thyroxine degradation.

Net irreversible removal of thyroxine from the "thyroxine space" would then be equivalent to T′ plus the fecal losses of unaltered thyroxine.

Attention in the present and in the preceding communication (6), has been centered upon the binding interactions which determine the reversible distribution of thyroxine. It is tempting to speculate upon the potential mechanisms by which these binding interactions could limit the rate of net thyroxine degradation *in vivo*.

Various possibilities suggest themselves:

1) The extrathyroidal "thyroxine space" averages ten per cent of body weight in euthyroid humans (19). This would imply that the static cellular/extracellular partition may always favor the extracellular phase and that only trace amounts of cell-bound thyroxine [*i.e.*, $(\mathrm{TBC})_{C}\,\mathrm{Tx}$] may be present at any single moment. Nonetheless, since cellular "uptake" must precede cellular disposition,[12] and since "uptake" is determined by the quantitative interactions between $(\mathrm{TBC})_{C}$, $(\mathrm{TBC})_{EC}$, and Tx, rate-limiting roles in the formation of T′ must be assigned to the static concentrations of all three.

2) At any value for the binding equilibrium between $(\mathrm{TBC})_{C}$, $(\mathrm{TBC})_{EC}$, and Tx: a) If rates of cellular degradation exceed those of cellular "uptake," then cell-binding [*i.e.*, $\mathrm{Tx} + (\mathrm{TBC})_{C} \rightleftharpoons (\mathrm{TBC})_{C}\,\mathrm{Tx}$] may limit net thyroxine turnover; b) If rates of cellular "uptake" exceed those of cellular degradation, then the rate at which T′ is formed may delimit the rate at which Tx is displaced from $(\mathrm{TBC})_{EC}$ onto $(\mathrm{TBC})_{C}$. The continued shift to the right would constitute a mechanism whereby cellular degradation of thyroxine could regulate the extracellular turnover of thyroxine.

3) Alternatively, despite a fixed cellular/extracellular distribution, net thyroxine degradation might be delimited by the turnovers of $(\mathrm{TBC})_{C}$ or $(\mathrm{TBC})_{EC}$, if: a) $(\mathrm{TBC})_{C}\,\mathrm{Tx}$ is degraded as an intact unit, or b) $(\mathrm{TBC})_{EC}\,\mathrm{Tx}$ enters the cell as an intact unit, or c) Tx is made available to the cell

[12] A relationship between "uptake" and subsequent disposition may also exist at subcellular levels. Klemperer has described a mitochondrial preparation wherein the uncoupling of certain oxidative phosphorylations by mitochondria *in vitro* (36) is directly correlated with the prior mitochondrial "uptake" of thyroxine from the suspending medium (37).

as a consequence of the disruption of $(TBC)_{EC}$ at the cell surface. The metabolism of thyroxine would thus be linked to the metabolic fate of the thyroxine-binding components.

To date, the diverse possibilities have not been explored in the intact animal. It does not seem unlikely that they are all concurrently operative in determining the net transcellular exchange of thyroxine. However, for the time being, they must be regarded as hypothetical extrapolations of more limited data, and as such, be subjected to future experimental documentation or refutation.

SUMMARY

1. The thyroxine-binding proteins of diverse human extracellular fluids have been localized by zone-electrophoretic techniques.

2. *In vitro* experiments have been conducted to assess the significance of extracellular proteins in the partition of thyroxine between cellular and extracellular phases. Model systems have been devised with surviving slices of liver, heart, and kidney cortex and with suspending media containing human plasma fractions of varied thyroxine-binding potencies.

3. It has been demonstrated that "uptake" of thyroxine by tissues *in vitro* is not dependent upon cellular metabolic activity, and that thyroxine distribution, in such systems, can be formulated in terms of a reversible binding equilibrium between cellular and extracellular thyroxine-binding components.

4. Possible implications of the data for the net transcellular flux of thyroxine *in vivo* have been discussed.

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SUBSTANCES RELEASED FROM THE SKIN FOLLOWING THERMAL INJURY. I. HISTAMINE AND PROTEINS [1]

By SOL ROY ROSENTHAL, CHARLES SAMET, RICHARD J. WINZLER, AND SELWYN SHKOLNIK

(From the Institution for Tuberculosis Research of the University of Illinois and the Departments of Preventive Medicine and Biochemistry, University of Illinois College of Medicine, Chicago, Ill.)

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The nature of the substances released from the skin following burning is not known. The majority of the studies concerned with this problem have involved analyses of blood or the lymph draining the burned part, and have been complicated by the complex composition of the blood and lymph and the complex interrelation between different tissues. The present studies represent an attempt to simplify the problem by employing excised skin and collecting the materials diffusing into a saline solution following exposure to hot water.

METHODS

Preparation of diffusates

Mongrel dogs were anesthetized with Nembutal® (30 mg. per kilo) and the skin of their backs was clipped with an Oster No. 40 hair clipper. By the use of an electrically driven dermatome [2] set at .015 inch, split thicknesses of skin [3] 3 in. wide and up to 12 in. long were obtained. The slices were placed immediately on saline-soaked gauze, cut up into 3-cm. squares, and secured with the cut surface inward about one end of glass cylinders, 2.4 cm. in diameter (Figure 1). The cut surface was in continuity with the inside of the cylinder. Before the burning, the cut surface was washed three times with 5 to 10 ml. of saline, and after the final washing, 2 ml. of saline were allowed to remain inside of the cylinder. Burning was done in hot water contained in a tray which held a U-shaped metal rack that supported the tubes in such a manner that only the skin was submerged in water (Figure 2). After burning at various temperatures and for various periods of time, the fluid was allowed to remain in the cylinder for 5 minutes while submerged in water at 37°C. This fluid was then collected, examined separately or pooled. Control samples were obtained from unburned skin following immersion for 5 minutes at 37°C. All experiments were complete within a half hour after the skin sections were prepared. Cooling of the skin with ice was avoided. The pooled material was immediately frozen and was kept in this state or was lyophilized for future use.

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and University of Illinois, NR 114-161.

[2] Brown electro-dermatome, Zimer Mfg. Co., Warsaw, Indiana.

[3] The cut of the skin as noted by microscopic section was slightly below the rete pegs and included about half of the reticulum layer of the cutis.

RESULTS

The following are some of the components identified in the diffusates:

Histamine

The histamine content of the diffusates was determined essentially by the method of Schultz and Dale (1, 2) using guinea pig ileum suspended in a 4-ml. bath. All values are expressed as micrograms of histamine per sample following the burning of a 4.5 sq. cm. piece of skin. With rare exceptions, a minimum of 3 individual samples was averaged for each experimental condition. Corrections were made for volume changes due to evaporation during heating.

Figure 3 shows the histamine released into the diffusate after exposure to various temperatures for 30 seconds to 20 minutes. It is seen that there

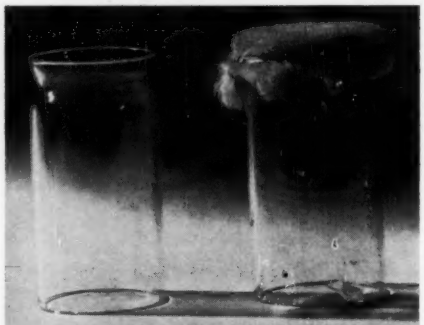

FIG. 1. GLASS CYLINDERS WITH AND WITHOUT SKIN IN PLACE (⅞ SIZE)

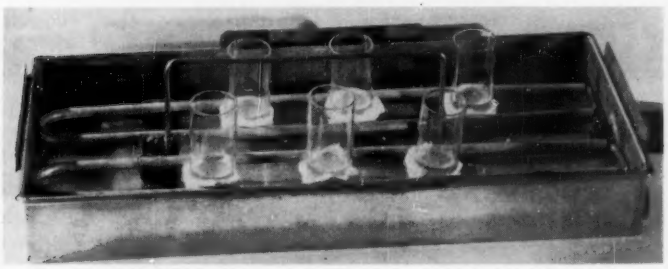

FIG. 2. RACK HOLDING SKIN PREPARATIONS IN PLACE
Burning of skin is accomplished by submerging skin in hot water of pan.

is a direct relationship between the temperature and time of burning and the extent of histamine release for the first two minutes of burning. Detectable amounts of histamine were released after 15 seconds of burning at 96°C and 85°C, 45 seconds at 75°C, and 2 minutes at 55°C. Only trace amounts of histamine were released after exposure to 47°C for periods of 1 to 10 minutes (in one of six experiments, .004 γ of histamine was released after 1, 3 and 5 minutes of burning). At temperatures of 85°C and 96°C for 5 minutes and more of burning, the values fall below the amounts obtained at 65°C or 75°C.

The non-specificity of the guinea pig ileum for histamine raised the possibility that the active substance was a compound other than histamine. That the substance in question was histamine was supported by the fact that the action on the guinea pig intestine was prevented by first adding the antihistamine, thymoxyethyldiethylamine (4 μgm. per ml.), to the bath. Contraction of the muscle strip caused by the diffusate was not inhibited by atropine, thus eliminating acetylcholine as the active component. Intravenous injection of the diffusates in dogs produced a fall in blood pressure equivalent to the amount of histamine present in the diffusates, this effect was also inhibited by thymoxyethyldiethylamine (40 mg. per kilo) subcutaneously one-half hour before testing.

The non-dialyzable portion of diffusates, dialyzed 24 hours against distilled water, failed to cause a drop of blood pressure in the dog.

Proteins

The amount of protein released in the diffusate was measured by the biuret method (Mehl [3]). Values are expressed as milligrams per sample (4.5 sq. cm. of skin) corrected by the values obtained from the unburned control skin. Each

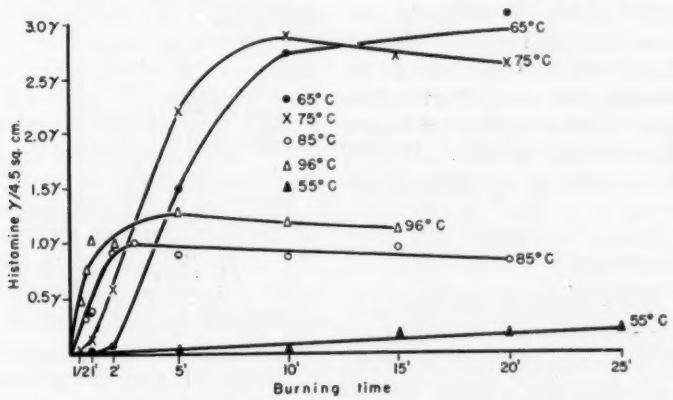

FIG. 3. HISTAMINE RELEASED FROM BURNED DOG SKIN AFTER EXPOSURE TO VARIOUS TEMPERATURES FOR VARYING PERIODS OF TIME
Each point is the average of at least 3 separate experiments.

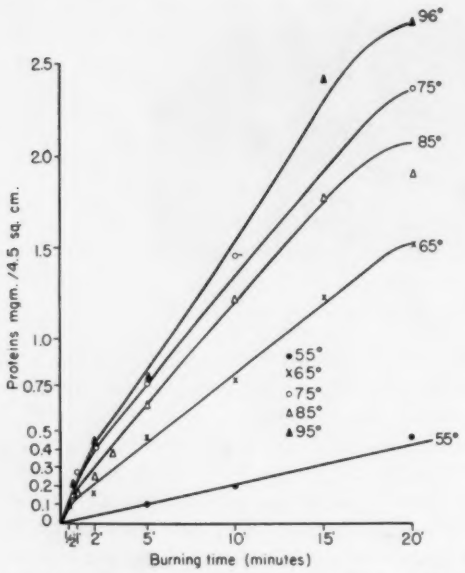

FIG. 4. PROTEIN RELEASE AFTER EXPOSURE TO VARIOUS TEMPERATURES FOR VARYING PERIODS OF TIME (BIURET METHOD)

Each point is the average of at least three separate experiments.

point on the curves (Figure 4) is an average of at least three separate experiments.

Figure 4 shows the relation of temperature and duration of burning to the amount of protein released. The amount of protein released from the dog skin was directly related to the temperature and the duration of injury (up to 20 min.). For temperatures of 75°C, 85°C and 96°C, the protein values approximated each other. After 20 minutes of burning at these high temperatures, the rate of protein release decreases. Under these conditions, coagulation was noted in the diffusates especially for those exposed to 96°C. At 47°C detectable protein was noted in only one of six experiments.

Nature of protein released

The question of the nature of the protein or protein split products released into the diffusate of control and burned skin was examined by several methods. The diffusates were collected from 20 to 100 similar experiments, the samples pooled, dialyzed for 48 hours against distilled water, lyophilized, and the residue weighed.

The results of these determinations are shown in column 2 of Table I. The amount of nondialyzable material increases from 0.27 mg. per sample (4.5 sq. cm. of skin) in the control to 13.6 mg. per sample in the skin exposed to 96°C for 15 minutes. The values obtained by this method are appreciably higher than those obtained by the biuret method employing single samples (Figure 4). The reason for this difference is not clear.

The electrophoretic behavior of the materials was examined after dialysis and lyophilization. Electrophoresis was carried out at pH 8.6 in 0.1 M veronal buffer at 10°C, using the Antweiler microelectrophoresis apparatus and photographing the Schlieren patterns.

The control sample showed the presence of three major components with mobilities corresponding to serum albumin, β globulin, and a very small amount of a protein with the mobility of γ globulin (slow component). All burned samples showed markedly decreased proportions of the fast moving components and increased amounts of components having the low mobility corresponding to that of γ globulin. In some cases this slow component appeared as a single electrophoretic component, whereas in other cases it was distinctly heterogeneous. Some typical electrophoretic patterns are shown in Figure 5. Electrophoretic results are summarized in column 3 of Table I. These data show that the protein liberated into the diffusate after exposure to heat is largely of a type having a very low electrophoretic mobility, and differs from the proteins present in the control samples.

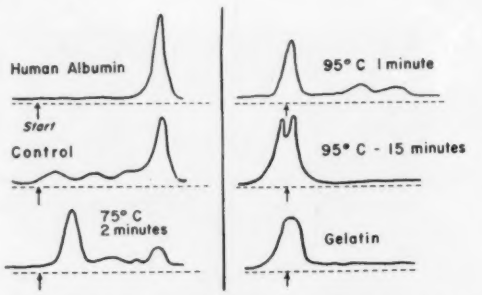

FIG. 5. ELECTROPHORETIC PATTERNS OF DIFFUSATES FROM BURNED SKIN AND CONTROLS

Antweiler microelectrophoretic apparatus at pH 8.6 in 0.1 M veronal buffer at 10°C. Starting boundary indicated by arrows. Time, 10 minutes. Movement to the right is toward the anode.

TABLE I

The nature of the proteins released from burned dog skin *

1	2	3		4	5	6	7	8	9
Proteins	Protein per sample (*mg. dry wt.*)	Micro-electrophoresis Comp.	%	Hydroxy proline %	Gelatin† %	Hexose %	Hexose in non-gelatin protein‡ %	Sialic acid§ %	Sialic acid in non-gelatin protein %
Control	0.27	Alb-α glob.	50	0.15	0.6	1.82	1.82	3.51	3.51
		β glob.	40						
		γ glob.	10						
75° C—2 min.	—	Alb-α glob.	30						
		β glob.	10						
		γ glob.	60						
96° C—1 min.	0.84	Alb-α glob.	40	1.45	10.8	3.84	4.3	3.09	3.29
		β glob.	10						
		γ glob.	50						
96° C—15 min.	13.6	Alb-α glob.	5	10.3	84.7	1.29	5.1	0.89	4.95
		β glob.	0						
		γ glob.	95						
Gelatin	—	Alb-α glob.	0	12.0	100.0	0.58		0.16	
		β glob.	0						
		γ glob.	100						
Serum albumin	—	Alb-α glob.	100	0.07	0	0.1		0.10	
		β glob.	0						
		γ glob.	0						

* Each value is the average of replicate determinations on two separate preparations which were usually in close agreement.

† Calculated on the basis that albumin contains 0.07 per cent hydroxyproline and gelatin contains 12.0 per cent.

‡ Calculated on the basis that gelatin contains 0.58 per cent carbohydrate.

§ Referral to a crystalline sample of "sialic acid" obtained from Blix at Upsala, Sweden.

It appeared possible that the slow moving components appearing in the diffusate might be gelatin, produced by the thermal degradation of collagen. Electrophoretic studies on gelatin (Table I) indicated that gelatin migrates very slowly in the microelectrophoresis apparatus. Further evidence that gelatin is the major protein in the diffusates from burned skin was obtained by carrying out determinations of hydroxyproline in the proteins isolated from the diffusates. Since collagen is the only tissue protein which contains large amounts of hydroxyproline, an estimate of the gelatin content of the fractions can be calculated from their hydroxyproline content.

Determinations of hydroxyproline were carried out by the method of Neuman and Logan (4). Calculation of the gelatin content of the proteins in the diffusates was made on the basis that albumin contained 0.07 per cent hydroxyproline and that gelatin contained 12 per cent. The results of these analyses, given in columns 4 and 5 of Table I, show that the control samples contained little hydroxyproline corresponding to less than 0.6 per cent gelatin. The samples heated at 96°C for 1 minute contained considerable gelatin and the samples heated at 96°C for 15 minutes consisted largely of this protein. Consequently, it can be concluded that a major part of the protein released into the diffusate upon exposure to heat is due to the thermal decomposition of collagen.

It is clear, however, that gelatin was not the only protein released from the tissue on burning. Were this the case, the gelatin content of the 96°C —1-minute samples should be 68 per cent, and of the 96°C—15-minute samples, 98 per cent, based on the increased protein content of the samples (column 2 of Table I). Consequently, other proteins were released as a result of heating, since the hydroxyproline content was less than that expected if all of the increased protein were gelatin.

It appeared possible that there might have been a depolymerization of mucoproteins and mucopolysaccharides of the skin as a result of thermal injury, and that some of these components might appear in the diffusates. In order to investigate this possibility, determination of the carbohydrate

content of the proteins isolated from the diffusates was carried out using the orcinol method of Sørensen and Haugaard (5). The results obtained are shown in column 6 of Table I. Since gelatin contains only 0.58 per cent carbohydrate, it is clear that a considerable amount of carbohydrate-rich protein was liberated into the diffusate as a result of heating. By subtracting the carbohydrate content of the gelatin, it is possible to calculate indirectly the carbohydrate content of the non-gelatin proteins present in each fraction. The results of this calculation are shown in column 7 of Table I. There is present protein with an average carbohydrate content of 4.3 per cent in the 96°C for 1-minute, and 5.1 per cent in the 96°C for 15-minute samples. These values are sufficiently high to indicate that carbohydrate-rich proteins are liberated as a result of thermal injury.

Further evidence that glycoproteins are liberated was obtained by assaying the proteins isolated from the diffusates for "sialic acid," a compound of unknown structure frequently associated with glycoproteins (Winzler [6]). For this purpose the p-dimethylamino-benzaldehyde method as described by Werner and Odin was used (7). The results of these analyses are shown in column 8 of Table I. The "sialic acid" content of the non-gelatin proteins is given in column 9 of Table I. These data also show that proteins relatively rich in "sialic acid" are released into the diffusate following thermal injury.

DISCUSSION

A major advantage of the method described is that it permits the direct detection of substances released from the skin as a result of burning or other stimuli. This method was first described in conjunction with the studies done on the mechanism of pain by Rosenthal and Minard (8). By this method it was shown that mechanical, electrical or thermal stimulation of the epidermis of the skin was followed by release of histamine in direct relation to the degree of the stimulus (9). The present modification uses an electrically driven dermatome which makes it possible to obtain a uniform split thickness of skin and facilitates the obtaining of large skin samples. Since much pressure is applied in obtaining these skin samples, the area is practically bloodless.

Histamine or a histamine-like substance has been found in the blood of shoats, pigs, guinea pigs, and humans following burning (10–12). It is likely from the present and other studies that skin is one source of this histamine. Harris (13), for example, found as much as 10 mg. of histamine per kilogram of human skin.

Following burning of the skin, proteins and protein split products are released from the skin. These materials are undoubtedly absorbed into the general circulation in burned animals. For example, a slow moving component which migrates with half the speed of gamma globulin was found in the lymph draining the scalded leg of calves but not in the serum by Perlmann, Glenn, and Kaufman (14). In the light of the present experiments it appears likely that this component was gelatin. A marked increase in nitrogenous material is noted in the blood following burning, both in experimental animals by Braasch, Bell, and Levenson (15) and in human beings by Taylor, Levenson, Davidson, and Adams (16). However, the nature and the source of this material has not yet been determined (Walker [17] and Rosenthal and McCarthy [18]).

There are two phases during which proteins and protein split products are released from the skin following scalding. The initial phase extends over the burning period while the second phase covers the period following the burning. The results described in this paper refer to the initial phase only. It has been shown by Ungar and Damgaard (19), using skin slices of the guinea pig, that the release of protein split products during the initial phase is not due to proteolytic action. The process proceeds at a fast rate at temperatures at which known proteases are destroyed and, furthermore, enzymatic inhibitors such as $CuSO_4$ and $HgCl_2$ fail to inhibit the release of protein products.

During the secondary phase of burning proteolysis is probably enzymatic in nature. It is inhibited partially by anti-proteases such as soy bean inhibitor and sodium salicylate (19).

SUMMARY

A method was developed for obtaining diffusates from excised dog skin after *in vitro* exposure to burning (scalding). It was found that exposure to heat caused a liberation of histamine or histamine-like substance into the diffusates. The amount of

histamine liberated depended upon the time and the temperature to which the excised skin was exposed to the injury. The greatest amount of histamine was released after exposure to 65°C and 75°C for 20 minutes. The lowest temperature consistently causing histamine release was 55°C for 2 minutes. The release of protein was increased with the time and temperature of burning. Determination of the hydroxyproline content and the electrophoretic behavior of such preparations indicated that the major portion of the proteins released, particularly at high temperatures and for prolonged periods, resembled gelatin. In addition, smaller amounts of other proteins containing appreciable amounts of hexose and "sialic acid" were released.

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CHEMICAL AND BIOLOGICAL ALTERATIONS INDUCED BY IRRADIATION OF I^{131} LABELED HUMAN SERUM ALBUMIN

By ROSALYN S. YALOW AND SOLOMON A. BERSON

(From the Radioisotope Service, Veterans Administration Hospital, Bronx 68, N. Y.)

(Submitted for publication July 13, 1956; accepted September 14, 1956)

It has been reported (1) and confirmed (2) that I^{131}-labeled human serum albumin may be heterogeneous with respect to its rate of metabolism *in vivo*. However, some preparations appear to be homogeneous and show a constant rate of degradation equal to the slowest rate of degradation observed in the heterogeneous preparations (1). This suggests that the more rapidly degraded components of the latter represent protein fractions damaged during the iodination procedure rather than intrinsically different proteins present in the original unlabeled material (1).

The inherent sensitivity of proteins to denaturation by various chemical and physical agents encountered during manipulations *in vitro* in general, and in labeling procedures in particular, has been well appreciated. However, while changes in cation and anion-binding capacity, ultraviolet absorption, and amino acid composition of serum albumin following x-irradiation have been reported (3), possible effects of self-irradiation of I^{131} labeled albumin on the rate of metabolism *in vivo* have not been studied. Recent experience with radiation-induced alterations of insulin-I^{131} and the protection against such alterations offered by the presence of serum albumin (4) led to the present investigation of the effects of irradiation on albumin-I^{131}.

METHODS

Human serum albumin [1] was labeled with I^{131} according to a method previously described (5). Appropriate amounts of carrier I^{127} were added so as to yield an average of less than one iodine atom per protein molecule. Four different lots of iodinated albumin were employed in the present study. After an iodination period of about five minutes the solutions were dialyzed at 4°C against large volumes of distilled water, with frequent changes, for about 7 hours. Three of the lots were then passed through Seitz filters into sterile vials. Aliquots of each lot were diluted with distilled water or fortified with non-labeled albumin as necessary to yield final protein concentrations of 200 μg. per ml., 5000 μg. per ml. or 50,000 μg. per ml. at a pH of 6.0. Half of the solution at each concentration was then irradiated immediately in a General Electric x-ray machine, peak energy 1 mev, at a dose rate of 1500 rad per min. for 30 minutes; the remaining half of each solution (control) was treated in an identical fashion except for the irradiation. Immediately after irradiation, sufficient non-labeled albumin was added to all control and irradiated solutions to bring protein concentrations up to 50 mg. per ml. in order to minimize the effects of further irradiation from the self-contained I^{131}.[2] In the case of the fourth lot, the experimental solution was irradiated by I^{131} added as iodide in a concentration of 2 mc. per ml. to a solution of albumin-I^{131} containing 200 μg. per ml. albumin.[3] After almost 5 days both the experimental and control portions were again dialyzed against water until the non-precipitable radioactivity was less than 1 per cent. Non-radioactive albumin was then added to both solutions in sufficient amounts to yield final protein concentrations of 50 mg. per ml.

Solutions were refrigerated for at least three days, while samples were checked for sterility by culture, and were then administered intravenously to hospitalized patients without proteinuria or known abnormalities of protein metabolism. Serum albumin concentrations were normal in all subjects. Blood samples were taken at frequent intervals and complete 24-hour urinary collections were made except during the first day when two collections were made. Assays of radioactivity were performed in a 5-ml. capacity well-type scintillation counter with a sensitivity of 1.00×10^6 counts per minute per μc. I^{131} above a background of 200 counts per minute.

In addition to the albumin-I^{131} prepared for studies *in vivo*, solutions of albumin-I^{131} were prepared at concen-

[1] Cutter albumin lot No. E 4172. This preparation revealed an α_1 protein content of about 2 per cent on free electrophoresis.

[2] The maximum irradiation received by any of these solutions from the I^{131} present was calculated to be about 500 rad prior to final addition of non-labeled albumin and less than 2000 rad total before administration. Since the iodoalbumin was prepared and maintained in solutions of 5 to 10 ml. the small amount of β-particle energy dissipated outside the solution was more than compensated for by the γ-ray energy absorbed in the solution. Therefore, the factor of 120 gm. rad per μc. I^{131} destroyed was employed in the calculations (6).

[3] The calculated radiation doses received by the experimental and control solutions of this lot were about 84,000 rad and 4000 rad, respectively.

trations of 20 μg. per ml. and were subjected to x-irradiation at dosage levels of 45 to 135 kilorad for electrophoretic analysis.

The reduction of ferricyanide ion in the presence and absence of p-chloromercuribenzoate was employed as a test for reactive sulfhydryl groups. Irradiated or control albumin solutions at initial concentrations of 200 μg. per ml. were concentrated five fold by freeze-drying prior to the ferricyanide tests. The test solutions were then incubated for one hour with and without p-chloromercuribenzoate (0.16×10^{-2} molar). Following the addition of potassium ferricyanide (0.7×10^{-3} molar) the solutions were incubated for another 15 minutes. Ferric sulfate (4.0×10^{-3} molar) in Duponal was then added. In the absence of p-chloromercuribenzoate the presence of freely reactive SH groups is evidenced by the appearance of a blue color (Prussian blue) within five minutes which reaches its maximum in about 15 minutes (early reaction). In the presence of p-chloromercuribenzoate, the color first begins to appear in about two hours (delayed reaction).

Aliquots of control and irradiated solutions were added to human plasma and subjected to electrophoresis and hydrodynamic flow on paper strips according to methods described previously (7).

RESULTS

Chemical effects of irradiation

Ten per cent cold trichloracetic acid precipitated 96 per cent to 98 per cent of radioactivity in the control solutions and 94 to 98 per cent in the x-irradiated solutions. The more accurate technique of short run paper electrophoresis showed 3 per cent of the total radioactivity present as iodide in the irradiated solutions and less than 1 per cent in the control solutions (Figure 1). Sufficient carrier iodide^{127} was added prior to electrophoresis to saturate the anion binding capacity of the albumin.

Aside from the appearance of small amounts of iodide^{131}, detectable abnormalities were not noted on electrophoresis of the x-irradiated solutions employed in the biologic studies. However, in the lot of albumin irradiated with I^{131} a small fraction of the radioactivity appeared to migrate with all the globulins. A similar phenomenon has been observed previously with irradiation damaged insulin-I^{131} (4, 7). The absence of these migrating components in the x-irradiated albumin-I^{131} solutions does not, however, indicate any qualitative difference in the effects of the two types of radiation employed, since the total radiation dose administered was almost twice as great in the lot irradiated with I^{131}. When albumin-I^{131} in concentrations of 20 μg. per ml. was irradiated with x-rays at dose levels of 45 to 135 kilorad, changes similar to those observed in the I^{131} irradiated solutions became readily apparent on electrophoretic analysis (Figure 2 a–d). At the highest radiation dose level virtually all the albumin-I^{131} was altered and there appeared radioactive products which migrated indiscriminately with all the serum proteins. A considerably larger amount of iodide^{131} was also present in these solutions as was evident in short run paper electrophoresis (Figure 2e). An apparent slightly greater mobility of the albumin-I^{131} than the stained serum albumin (Figure 2 a–d) is an artifact introduced by the resolving time of the recording system. The peaks of radioactivity and albumin stain were found to correspond exactly when the strips were sectioned and assayed in a scintillation counter.

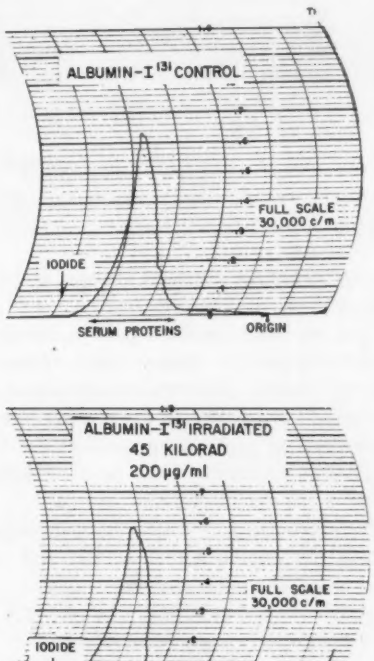

FIG. 1. RADIOACTIVITIES OF PAPER STRIPS FOLLOWING ONE AND ONE-HALF HOURS OF ELECTROPHORESIS COMBINED WITH HYDRODYNAMIC FLOW

Origin indicates site of application of solutions. (Phosphate buffer, ionic strength 0.1; constant voltage 250 V, Whatman 3 MM paper; migration to the left.)

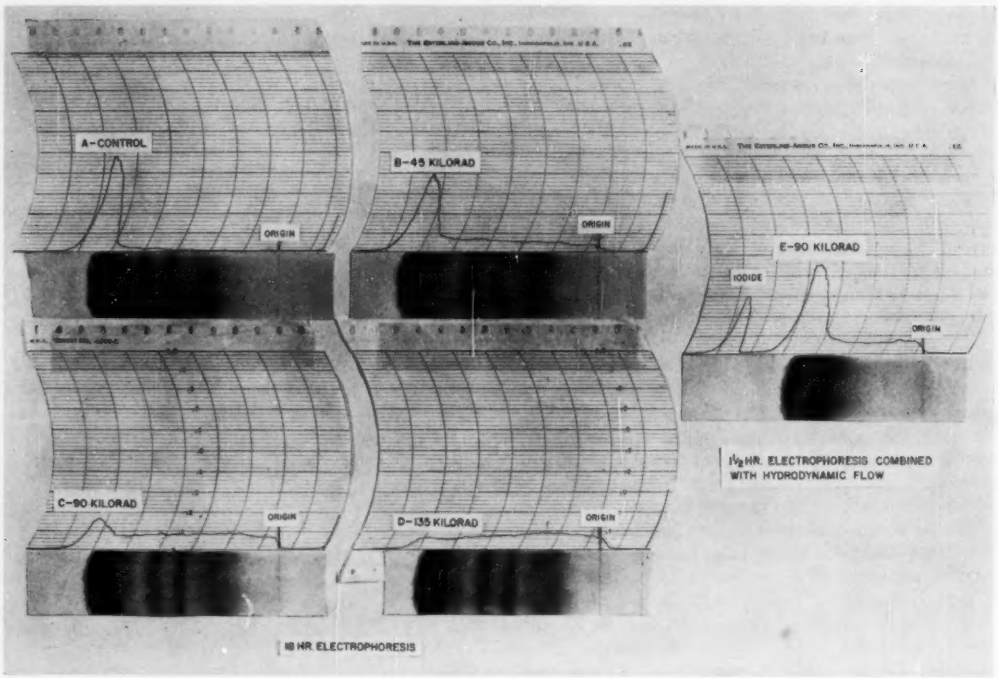

FIG. 2. PAPER STRIP RADIOELECTROPHORETOGRAMS OF ALBUMIN-I^{131} SOLUTIONS IN PLASMA

Albumin-I^{131} solutions were subjected to irradiation doses as indicated at albumin concentrations of 20 μg. per ml. Full scale reading 30,000 counts per minute on all strips.

The nature of the chemical changes accompanying these effects are not completely known. However, one important alteration, the splitting of the disulfide linkages of albumin with reduction to sulfhydryl groups, has been demonstrated by the reduction of ferricyanide in the absence but not in the presence of p-chloromercuribenzoate (Table I). These observations have been confirmed repeatedly with non-labeled albumin as well. This radiochemical change has also been demonstrated following the irradiation of insulin and is considered in greater detail elsewhere (8).

TABLE I

Prussian Blue reaction

	Without p-chloro-mercuri-benzoate	With p-chloro-mercuri-benzoate
Irradiated water	0	0
Control albumin-I^{131}	0	0
Irradiated albumin-I^{131}	Early	Delayed

Biologic effects of irradiation

Following administration of solutions which received approximately 45,000 rad from x-rays at a concentration of 200 μg. albumin per ml., a large fraction of the labeled protein was degraded rapidly during the first few days, as evidenced by the excretion of large amounts of non-protein bound radioactivity in the urine (Figure 3) and the rapid disappearance of protein bound radioactivity from the plasma (Figure 4) in contrast to the control non-irradiated material. The rate of removal from the plasma of the moiety remaining after the first week or so was also greater with irradiated than with control solutions although there was a tendency for some of the individual curves in the former group to flatten out, indicating the presence of protein fractions less severely affected by the irradiation.

When solutions containing 5000 μg. protein per ml. received 45,000 rad, alteration of the protein, as manifested by rapid degradation, was definitely

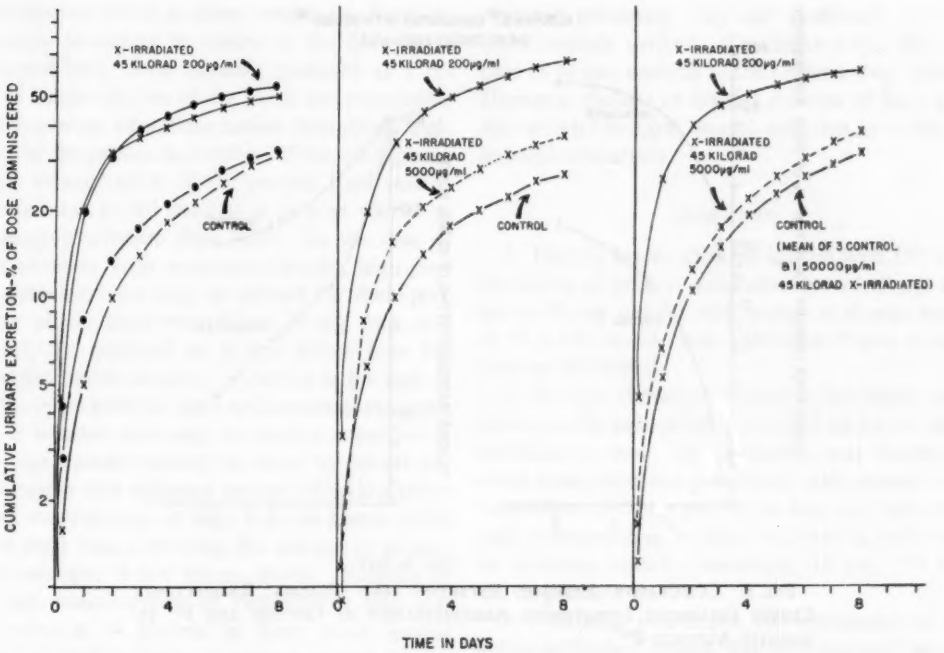

FIG. 3. CUMULATIVE URINARY EXCRETION FOLLOWING INTRAVENOUS ADMINISTRATION OF CONTROL AND X-IRRADIATED ALBUMIN-I^{131} SOLUTIONS TO NORMAL HUMAN SUBJECTS

In the third frame, the curves from the three subjects receiving control albumin-I^{131} solutions and the one subject who had received the albumin-I^{131} irradiated at a concentration of 50 mg. per ml. were virtually indistinguishable.

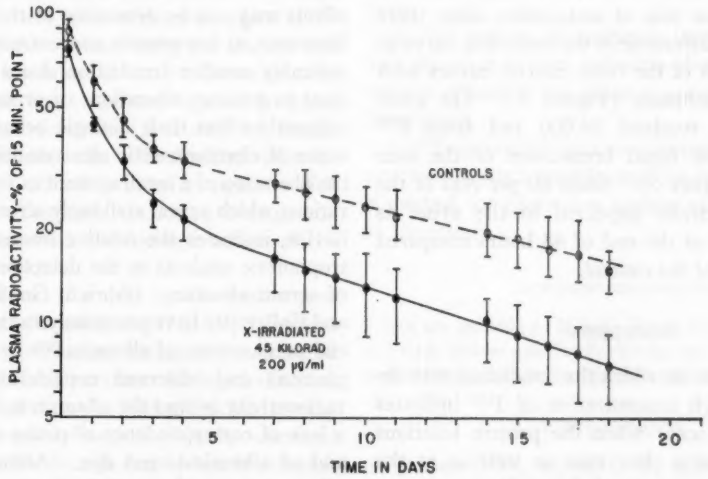

FIG. 4. MEAN PLASMA CONCENTRATION OF RADIOACTIVITIES IN SIX SUBJECTS RECEIVING CONTROL ALBUMIN-I^{131} AND FOUR SUBJECTS RECEIVING IRRADIATED ALBUMIN-I^{131}

Horizontal bars indicate extreme range of values for each point.

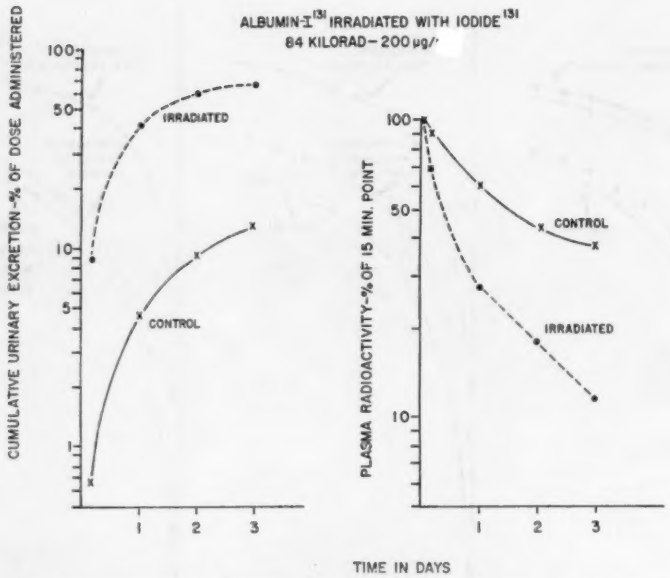

FIG. 5. CUMULATIVE URINARY EXCRETION AND PLASMA RADIOACTIVITY CURVES FOLLOWING INTRAVENOUS ADMINISTRATION OF CONTROL AND I^{131} IRRADIATED ALBUMIN-I^{131}

much less striking than in the case of the more dilute solutions, but there still appeared to be a definite effect (Figure 3). In the single study employing a concentration of 50,000 µg. albumin per ml., 45,000 rad seemed to have had no detectable effect on the rate of metabolism since there was no definite difference in the excretion curve as compared to that of the three control curves with this lot of iodoalbumin (Figure 3). The albumin-I^{131} which received 84,000 rad from I^{131} showed the most rapid breakdown of the four lots studied (Figure 5). Some 60 per cent of the injected radioactivity appeared in the urine as protein-free I^{131} at the end of 48 hours compared to 9.6 per cent for the control.

DISCUSSION

The experiment in which the irradiation was delivered by a high concentration of I^{131} indicates that alterations occur when the protein solutions are irradiated at a slow rate as well as at the higher dose rates obtained from the x-ray machine. The x-ray doses employed here (45,000 rad) are approximately equivalent to the radiation absorbed in one week by solutions initially containing 0.8 mc. I^{131} per ml. I^{131} labeled proteins having about this concentration of radioactivity are distributed commercially and are frequently prepared by investigators in their own laboratories. When protein concentrations are high, irradiation effects may not be detectable at these dose levels. However, at low protein concentrations, even considerably smaller irradiation doses may be sufficient to produce alterations of proteins which significantly affect their biologic behavior. The absence of electrophoretic abnormalities, apart from the liberation of a small amount of iodide, in preparations which reveal strikingly altered biologic behavior, indicates the relative insensitivity of electrophoretic analysis in the detection of alterations of serum albumin. Gabrieli, Goulian, Kinersley, and Collet (9) have previously examined commercial preparations of albumin-I^{131} by paper electrophoresis and observed considerable trailing of radioactivity behind the albumin in serum and also a lack of correspondence of peaks of radioactivity and of albumin-bound dye. Although the latter effect was not observed in the preparations employed here, the trailing observed by these authors may well have been a manifestation of irradiation damage.

Irradiation effects in dilute protein solutions are primarily secondary in nature at the dose levels employed here. Free radicals produced as a result of the irradiation of the water are responsible for the protein alterations rather than direct ionization of the protein molecules. Thus, an increase in the concentration of the protein itself results in a decrease in the fraction of protein altered at the same irradiation dose level. In the case of some proteins, such as serum albumin, high protein concentrations may be utilized for their protective effect, since even 25 to 50 mg. may still properly be regarded as a true tracer dose for metabolic studies in man. However in the case of certain other proteins such as hormones, antigens, etc., it is often necessary to employ solutions of very high specific activity in order to permit administration of a sufficient amount of radioactivity for subsequent assay in body fluids or tissue, while at the same time restricting the amount of protein to physiologic, if not tracer, levels. Because of the high concentration of radioactivity and low concentration of protein in these cases special measures must be taken to protect against the irradiation induced damage. In such instances it has been recent practice in this laboratory to remove rapidly, by dialysis, unbound I^{131} from the iodoprotein solutions and to follow immediately with the addition of sufficient amounts of serum albumin to yield final protein concentrations of 50 to 100 mg. per ml. Almost complete dialysis can be effected in a few hours if small volumes of solution (5 to 10 ml.) are dialyzed against frequent changes of large volumes (2 liters) of water, saline or other solutions. The added albumin serves as an unlabeled protective agent which minimizes any subsequent irradiation damage and does not interfere with the biologic action of the labeled proteins (4).

There is no intent to imply, by the studies presented here, that alteration of I^{131} labeled proteins induced by self-irradiation necessarily represents the major hazard to proteins during iodination procedures. Alterations in biologic behavior may be induced by overiodination (1), by chemical changes or by other factors even when irradiation effects are minimized by the use of low specific activity preparations. Variability in the rate of metabolism of albumin-I^{131} with preparations produced by different iodinating procedures has been reported previously (1) and confirmed recently (10); certain methods of iodination (1, 10) appear to be less noxious to the proteins than others. However, the use of labeled proteins of high specific activity requires special attention to radiation induced alterations.

SUMMARY

1. Human serum albumin labeled with I^{131} was irradiated at protein concentrations of 20 μg. per ml. to 50 mg. per ml. with x-rays at dosage levels of 45 to 135 kilorad and with iodide^{131} at a dosage level of 84 kilorad.

2. Biologic alteration induced by irradiation was evidenced by susceptibility to rapid metabolic degradation *in vivo*. This alteration was detectable when irradiation was performed with protein concentrations of 5 mg. per ml. or less and with dosages corresponding to those received in one week by solutions initially containing 0.8 mc. I^{131} per ml. or greater.

3. Following irradiation, the appearance of reactive sulfhydryl groups was demonstrated by reduction of ferricyanide in the absence but not in the presence of p-chloromercuribenzoate.

4. Anomalous migration of I^{131} labeled fragments on paper electrophoresis and liberation of I^{131} as iodide were also observed following irradiation.

5. The extent of alteration induced was a direct function of the radiation dose and was inversely related to the protein concentration during irradiation.

6. The potential radiation damage to I^{131} labeled proteins of high specific activity is discussed and a method for protection against radiation damage is suggested.

ACKNOWLEDGMENTS

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THE HEXOSAMINE CONTENT OF THE SERUM GLOBULINS IN NORMAL AND PATHOLOGICAL SERA[1]

By ALFRED JAY BOLLET[2] with the technical assistance of JURIJ ROZHIN and WILLIAM SIMPSON

(From the Department of Medicine of Wayne State University, College of Medicine, and Detroit Receiving Hospital, Detroit, Mich.)

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Considerable interest has been focused upon the glycoproteins present in serum. The hexoses and hexosamines associated with serum proteins have been found to be elevated non-specifically in a variety of pathologic processes (1–9). The seromucoid, a protein fraction particularly rich in carbohydrate, studied extensively by Winzler (2), also is increased in similar disease states (2, 10–15). The amount of carbohydrate in the alpha globulin fraction has been found to be elevated in tuberculosis (16), gout (4), rheumatoid arthritis and rheumatic fever (17), whereas the carbohydrate in the gamma globulin fraction has been reported to be increased in lupus erythematosus (5, 17).

The present study was undertaken to characterize further the alterations which occur in one of the carbohydrate components, hexosamine, in electrophoretically separated serum protein fractions and in seromucoid in a variety of disease processes, with particular emphasis on the rheumatic diseases, and following the administration of anti-inflammatory hormones.

METHODS

Blood samples were obtained in the fasting state from healthy blood donors and patients with a variety of rheumatic diseases, pneumonia, cirrhosis and hepatitis. The serum was separated within a short time after clotting, and kept frozen until used.

Hexosamine determinations were done by the method of Boas (18), which involves isolation on Dowex-50 columns after preliminary hydrolysis, followed by determination of the hexosamine by a modification of the Elson-Morgan method. Electrophoresis was done in veronal buffer pH 8.6, ionic strength .05, on Whatman No. 3 paper suspended horizontally in a vapor chamber. Runs were performed at approximately 15 m A and 200 V, for 15 hours. The protein content of each fraction was determined by heat fixation, staining with bromphenol blue, cutting into components and eluting the dye in .01 N NaOH. The percentage of the total dye in each component was multiplied by the total protein, determined by the biuret method using Armour's bovine albumin as standard. No correction was made for differences in dye binding by the different protein fractions. Determination of the hexosamine content of the electrophoretically separated protein fractions was done following separation of 0.1-ml. samples of serum. After fixing and staining, the paper was cut into appropriate strips and each component was hydrolyzed and determined individually. This is similar to the method used by Boas, Bollet, and Bunim (4). Appropriate control studies showed that no detectable loss of hexosamine occurred during any of these steps, and added standard solutions of hexosamine were quantitatively recovered. Duplicate electrophoretic runs and determinations of each component were done for both protein and hexosamine. The reproducibility for both protein and hexosamine content of each fraction on replicate electrophoretic separations was found to about ± 5 per cent.

The perchloric acid-soluble, phosphotungstic acid-precipitable fraction (seromucoid) was isolated by a modification of the method of Greenspan, Lehman, Graff, and Schoenbach (15). The phosphotungstic acid precipitate obtained from the perchloric acid filtrate was divided into two fractions. One was analyzed for protein content by the biuret method using a casein standard, and the other analyzed for hexosamine after hydrolysis in 1 N HCl for 15 hours.

RESULTS

Total serum hexosamine

Normal individuals were found to have serum hexosamine levels averaging 79.0 mg. per cent. Elevations of the total serum hexosamine were found in the diseases listed (Table I).

The ratio of total serum hexosamine to total serum protein (× 100) was found to average 1.02 in the normal individuals. In all of the diseases studied, the ratio of total hexosamine to total protein increased, the maximum being an average of 2.06 in the patients with rheumatic fever (Table

[1] Aided by grants from the Michigan Chapter of the Arthritis and Rheumatism Foundation, The Schering Corporation and the Research and Education Corporation of the Detroit Receiving Hospital.

[2] Markle Scholar in Medical Science.

I). This change occurred regardless of the direction of change of the total protein.

Hexosamine content of electrophoretically separated globulins

In order to define more precisely the observed changes in total serum hexosamine, analyses were made of each of the globulin fractions obtained after electrophoretic separation of the serum specimens on filter paper. In this part of the study, analyses were done on pools of sera, each containing equal amounts of sera from three patients with the same disease. Since each determination represents the average of duplicate electrophoretic runs on each pool, it is felt that the mean levels found have validity, but no attempt has been made to establish the range of variation for each globulin fraction in each disease. The amount of hexosamine in the electrophoretically separated serum albumin was found to be too small for accurate determination in the situations studied, and the details are therefore not included in this report.

In three pools of normal sera, the mean hexosamine content of the alpha-1 globulin averaged 17.5 mg. per cent, of the alpha-2 globulin, 25.0 mg. per cent, of the beta globulin, 17.4 mg. per cent, and of the gamma globulin, 24.7 mg. per cent. The amount of protein in each of these fractions was also determined. The ratio of hexosamine to protein (× 100) in the alpha-1 globulin averaged 4.3. In the alpha-2 globulin, the hexosamine to protein ratio averaged 4.1, in the beta globulin 2.9, and in the gamma globulin 1.8 (Table II).

In four pools of sera from patients with active rheumatoid arthritis, the amount of hexosamine in each globulin fraction was found to be increased, although the increment in the beta fraction was minimal. Comparison of the hexosamine levels to the protein content of each fraction showed that the hexosamine to protein ratio in alpha-1 globulin was considerably greater than in the normal pools, reaching 7.4, whereas in the other fractions changes in the hexosamine to protein ratios were of questionable significance.

Two pools of sera from patients with rheumatic

TABLE I

Total serum hexosamine concentration, total serum protein concentration, and ratio of hexosamine to protein in health and disease

Diagnosis	No. of cases		Total hexosamine mg. %	Total protein gm. %	$\frac{\text{Hexosamine}}{\text{Protein}} \times 100$
Normal	25	Range	65.8–107	6.34–8.45	0.95–1.08
		Mean	79.0	7.34*	1.02*
		S.E.M.	±1.84	±0.55	±0.14
Rheumatoid Arthritis	14	Range	81.6–147	5.09–9.20	1.21–2.26
		Mean	117	7.28	1.63
		S.E.M.	±3.93	±.28	±.077
			(p = >.001)		(p = >.001)
Rheumatic Fever	8	Range	97–193	5.28–9.41	1.48–2.99
		Mean	153	7.59	2.06
		S.E.M.	±10.8	±.49	±.184
			(p = >.001)		(p = >.001)
Systemic Lupus Erythematosus	4	Range	114–150	6.20–8.64	1.40–2.14
		Mean	126	7.47	1.71
Gout	3	Range	88.5–130	6.07–6.92	1.28–2.14
		Mean	104	6.52	1.62
Pneumonia	7	Range	105–179	6.28–7.84	1.43–2.42
		Mean	127	7.22	1.76
		S.E.M.	±9.79	±.21	±.124
			(p = >.001)		(p = .001)
Cirrhosis of Liver	4	Range	105–136	5.98–8.08	1.61–2.15
		Mean	125	6.69	1.89
Hepatitis	3	Range	86.9–111	7.88–8.30	1.05–141
		Mean	102	8.09	1.26

* Based on 11 cases.

TABLE II

Hexosamine and protein content of electrophoretically separated serum protein fractions. Each pool contained equal amounts of serum from three patients

Diagnosis	Hexosamine (*mg.* %)				Protein (*gm.* %)					$\frac{\text{Hexosamine}}{\text{Protein}} \times 100$			
	alpha-1	alpha-2	beta	gamma	alb.	alpha-1	alpha-2	beta	gamma	alpha-1	alpha-2	beta	gamma
Normal I	17.8	20.7	15.4	22.8	4.15	0.41	0.52	0.61	1.46	4.3	4.0	2.5	1.6
Normal II	16.5	23.6	16.8	25.4	4.10	0.40	0.67	0.64	1.44	4.1	3.5	2.6	1.8
Normal III	18.3	30.7	20.5	26.0	4.19	0.40	0.66	0.58	1.40	4.4	4.7	3.5	1.9
(Mean)	(17.5)	(25.0)	(17.4)	(24.7)	(4.15)	(0.40)	(0.62)	(0.61)	(1.43)	(4.3)	(4.1)	(2.9)	(1.8)
Rheumatoid Arthritis I	32.4	49.8	20.5	28.8	3.15	0.42	1.02	0.57	1.85	7.7	4.9	3.6	1.6
Rheumatoid Arthritis II	32.8	51.4	24.7	33.1	2.99	0.44	0.99	0.65	1.98	7.5	5.2	3.8	1.7
Rheumatoid Arthritis III	28.3	35.0	23.8	31.0	3.25	0.47	0.83	0.67	1.49	6.0	4.2	3.6	2.1
Rheumatoid Arthritis IV	20.6	29.5	18.1	40.4	2.45	0.36	0.50	0.58	2.03	8.5	5.9	3.1	2.0
(Mean)	(28.5)	(41.4)	(21.8)	(33.3)	(2.96)	(0.42)	(0.84)	(0.62)	(1.84)	(7.4)	(5.1)	(3.5)	(1.9)
Rheumatic Fever I	36.2	48.1	24.1	34.0	3.32	0.54	1.08	0.83	2.18	6.7	4.5	2.9	1.6
Rheumatic Fever II	30.5	39.6	21.7	30.3	3.22	0.43	0.88	0.82	1.84	7.1	4.5	2.6	1.6
(Mean)	(33.4)	(43.9)	(22.9)	(32.2)	(3.27)	(0.49)	(0.98)	(0.83)	(2.01)	(6.9)	(4.5)	(2.8)	(1.6)
Systemic L. E. I	24.0	37.7	24.9	49.9	2.97	0.42	0.73	0.75	2.85	5.7	5.2	3.3	1.8
Systemic L. E. II	36.8	40.0	15.4	26.2	1.68	0.56	0.79	0.53	1.61	6.6	5.1	2.9	1.6
(Mean)	(30.4)	(38.9)	(20.2)	(38.1)	(2.38)	(0.49)	(0.76)	(0.64)	(2.23)	(6.2)	(5.2)	(3.1)	(1.7)
Pneumonia I	27.9	44.8	20.4	35.0	3.34	0.44	1.04	0.68	2.08	6.3	4.3	3.0	1.7
Pneumonia II	44.3	45.7	25.4	38.0	2.06	0.40	0.62	0.59	2.63	11.0	7.4	4.2	1.4
(Mean)	(36.1)	(45.3)	(22.9)	(36.5)	(2.70)	(0.42)	(0.83)	(0.64)	(2.36)	(8.7)	(5.9)	(3.6)	(1.6)
Cirrhosis I	21.4	29.7	18.4	46.9	2.35	0.53	0.81	0.71	2.61	4.0	3.7	2.6	1.8
Cirrhosis II	16.8	24.0	16.6	56.0	2.20	0.34	0.62	0.55	2.58	4.9	3.9	3.0	2.1
(Mean)	(19.1)	(26.9)	(17.5)	(51.5)	(2.28)	(0.44)	(0.72)	(0.63)	(2.60)	(4.5)	(3.8)	(2.8)	(2.0)

fever showed significant elevations in the hexosamine content of the alpha-1, alpha-2 and gamma fractions, with slight increase in the beta globulin hexosamine. The hexosamine to protein ratios again showed a considerable increase in the alpha-1 globulin, but little change in the other fractions. Similar findings were noted in two pools of sera from patients with active systemic lupus erythematosus. The total gamma globulin was considerably increased in one of these pools, and a correspondingly increased gamma globulin-hexosamine was found, with no change in the hexosamine to protein ratio.

Pools of sera from patients with acute bacterial pneumonia showed changes very similar to those found in rheumatoid arthritis and rheumatic fever. In one of these pools there were marked increases in hexosamine content of the alpha-2 and beta globulins, without corresponding increases in protein in those fractions. This was the only pool which showed apparently significant increases in the ratio of hexosamine to protein in those fractions.

Sera from patients with decompensated Laënnec's cirrhosis were also studied, in view of the suggested relation between liver function and serum glycoprotein components (8, 14). The only significant deviation from the normal findings in these sera consisted of an elevation of gamma globulin with a corresponding elevation of gamma globulin-hexosamine, with no change in the ratio of hexosamine to protein (Table II).

Effect of anti-inflammatory hormones on hexosamine content of alpha globulins

A group of patients with rheumatic fever, gout and rheumatoid arthritis were studied before and during therapy with various anti-inflammatory hormones (Table III). The hexosamine content of the alpha-1 globulin fell during treatment in all instances. The protein content of the alpha-1 globulin fell when it was considerably elevated before treatment, but rose during therapy in one patient with rheumatic fever, and in the pool of three cases of rheumatoid arthritis. The ratio of hexosamine to protein in the alpha-1 globulin fell in all.

The hexosamine content of the alpha-2 globu-

lin fell with therapy in the patients with rheumatic fever, but did not change significantly in the other instances. A diminution in the protein content of the alpha-2 globulin also occurred in the patients with rheumatic fever; the ratio of hexosamine to protein in the alpha-2 globulin did not change significantly in any instance.

The perchloric acid soluble fraction (seromucoid)

Studies of this fraction were done on sera from individual patients and also on the pools of sera used for the electrophoretic separations.

Twenty-five normal sera showed a mean hexosamine content of the seromucoid of 5.69 mg. per cent; the biuret reaction revealed a protein content averaging 79.5 mg. per cent. The ratio of hexosamine to protein (× 100) in this fraction averaged 7.3 with these methods. Of the total hexosamine found in these sera, 5.8 per cent was found in the seromucoid.

Sera from patients with rheumatoid arthritis showed elevations in the protein content of the seromucoid averaging 121 mg. per cent; the hexosamine in this fraction was also elevated, averaging 11.6 mg. per cent. The ratio of hexosamine to protein in this fraction averaged 11.5. This ratio of hexosamine to protein varied from the normal range up to 16.9, and clinically there seemed to be a rough correlation between this ratio and the severity of the joint inflammation. Of the total hexosamine content of these sera, 9.7 per cent was found in this fraction (Table IV).

In active rheumatic fever, systemic lupus erythematosus and acute gouty arthritis similar changes were found, with increases in the ratio of hexosamine to protein (Table IV). As the disease process subsided, the protein and hexosamine content of the seromucoid fraction both fell toward the values found in normal subjects. The ratio of hexosamine to protein also decreased. The greatest increases in the amount of each constituent of the seromucoid were observed in some cases of acute rheumatic fever.

Eight patients with bacterial pneumonia also were studied, and similar increases in hexosamine and protein were found, with a rise in the hexosamine to protein ratio.

Eight patients with decompensated Laënnec's cirrhosis and three with acute viral hepatitis also were studied. The protein content of the seromucoid was found to be lower than normal, but in some instances there was considerable overlap into the normal range (Table IV). The hexosamine content of this fraction was not significantly reduced, resulting in a rise in the hexosamine to protein ratio.

Effect of anti-inflammatory hormones on the seromucoid fraction

Sixteen patients were studied before and after therapy with corticotropin (ACTH), cortisone or related steroids. The findings in the seromucoid fraction given in Table V are the mean values for the patients in each group before and after therapy.

TABLE III

Effect of therapy with anti-inflammatory hormones on hexosamine and protein content of alpha globulins

Disease	Status	Hexosamine mg. %		Protein gm. %		Hexosamine / Protein	
		alpha-1	alpha-2	alpha-1	alpha-2	alpha-1	alpha-2
Rheumatic Fever	Before Rx	35.3	56.5	0.41	1.19	8.6	4.8
	Hydrocortisone Rx	28.5	38.3	0.47	0.75	6.1	5.1
Rheumatic Fever	Before Rx	68.9	86.8	0.82	1.49	8.4	5.8
	Hydrocortisone Rx (2 weeks)	27.1	44.9	0.43	0.80	6.3	5.6
	Hydrocortisone Rx (5 weeks)	20.7	33.0	0.41	0.61	5.1	5.4
Gout	Before Rx	35.5	51.8	0.56	1.20	6.3	4.3
	ACTH Rx	25.0	50.0	0.54	1.16	4.6	4.3
Rheumatoid Arthritis (Pool)	Before Rx	28.3	35.0	0.47	0.83	6.0	4.2
	Prednisone Rx	25.3	36.5	0.51	0.95	4.9	3.8
Mean change with Rx		−17.1	−17.9	−0.08	−0.31	−2.7	−0.1

TABLE IV

Hexosamine and protein content of seromucoid fraction in normal and pathological sera

Diagnosis	No. of obs.		Hexosamine mg. %	Protein mg. %	$\frac{\text{Hexosamine}}{\text{Protein}} \times 100$	$\frac{\text{Seromucoid hexosamine}}{\text{Total hexosamine}} \times 100$
Normal	25	Range	3.76–8.45	47.5–107	5.55–9.02	5.39–7.96
		Mean	5.69	79.5	7.20	6.97
		S.E.M.	±0.26	±3.3	±.26	±.91
Rheumatoid Arthritis	16	Range	5.54–19.9	44.3–264	8.13–16.9	6.00–17.3
		Mean	11.6	121	11.5	9.7
		S.E.M.	±1.6	±15.2	±.76	±.80
			(p = >.001)	(p = .01)	(p = >.001)	(p = .05)
Rheumatic Fever	8	Range	10.5–25.6	134–313	7.4–12.1	8.1–14.2
		Mean	17.9	190	9.5	11.6
		S.E.M.	±2.13	±23.5	±.49	±.77
			(p = >.001)	(p = >.001)	(p = >.001)	(p = .02)
Pneumonia	8	Range	8.22–19.6	50.0–205	7.8–13.0	6.4–10.9
		Mean	9.14	116	9.0	8.2
		S.E.M.	±1.68	±16.9	±.61	±1.0
			(p = .05)	(p = .05)	(p = .01)	(p = .4)
Systemic Lupus Erythematosus	5	Range	6.24–12.5	65.3–167	9.3–16.6	5.2–10.7
		Mean	11.0	95.8	12.0	7.8
Gout	3	Range	7.26–17.1	50.4–128	10.2–14.4	7.7–13.2
		Mean	10.6	83.6	12.7	9.8
Cirrhosis	8	Range	2.31–12.6	33.8–89.3	7.5–16.4	4.3–9.3
		Mean	5.57	59.7	9.8	5.2
		S.E.M.	±1.27	±8.18	±1.2	±1.4
			(p = .4)	(p = .02)	(p = .05)	(p = .3)
Hepatitis	3	Range	4.02–6.02	43.1–69.0	8.5–10.9	4.4–7.0
		Mean	4.90	53.2	9.4	5.3

In five patients with rheumatoid arthritis, the total serum hexosamine and the hexosamine in the seromucoid fraction both decreased with hormone therapy. At the same time an increase occurred in the biuret reacting protein in the seromucoid. In seven patients with acute rheumatic fever, similar decreases in total serum hexosamine and seromucoid hexosamine occurred, but in these patients the protein content of the seromucoid, which was markedly elevated before therapy, fell with treatment. The fall in protein was proportionately less than the fall in hexosamine, however, and the hexosamine to protein ratio in this fraction fell toward normal. In a group of four patients with unrelated disease (acute gouty arthritis, acquired hemolytic anemia, systemic lupus erythematosus, and idiopathic myocarditis) the changes were similar to those found in the rheumatoid arthritis patients. In all instances, the ratio of hexosamine to protein in the seromucoid fraction was decreased after therapy.

TABLE V

Effect of therapy with anti-inflammatory hormones on hexosamine and protein in seromucoid fraction. (Figures given are mean values)

	Hexosamine mg. %	Protein mg. %	$\frac{\text{Hexosamine}}{\text{Protein}} \times 100$
Rheumatoid Arthritis (5 cases)			
Before therapy	13.4	103.	13.0
On therapy	11.9	130.	9.2
Rheumatic Fever (7 cases)			
Before therapy	17.3	183.	9.5
On therapy	9.9	126.	7.8
Non-rheumatic diseases (4 cases)			
Before therapy	12.1	106.	11.4
On therapy	8.8	112.	7.9

DISCUSSION

The range for normal total serum hexosamine found in this study is very similar to that reported by West and Clarke (1). The non-specific in-

crease found in a variety of diseases also duplicates the reports of others (1–4, 6). Our data indicate that the rise in total serum hexosamine in various diseases is accompanied by an increase in the ratio of hexosamine to total serum protein. An increase in the ratio of total protein-bound hexose to total serum protein has been described in a variety of diseases (7, 19), and an increase in the ratio of total hexosamine to total serum protein has been reported in rheumatic fever (6).

The largest increases in carbohydrate content of the serum proteins in most disease states have been found in the alpha globulins (4, 16, 17). Our data confirm these observations. In addition, it was found that an increase in the ratio of hexosamine to protein occurred in the alpha-1 globulin, in non-specific fashion. The ratio of hexosamine to protein in the other fractions, as determined by these methods, did not change significantly despite rather large increases in the amount of alpha-2 globulin in most of the diseases studied, and in the gamma globulins in some clinical states.

With suppression of the inflammatory process by means of hormone therapy, the amount of hexosamine in the alpha-1 globulin decreased, and the ratio of hexosamine to protein also fell. Changes in the amount of hexosamine in the alpha-2 globulin during therapy were accompanied by changes of similar magnitude in the protein content of that fraction, so that no significant alteration in the ratio of hexosamine to protein occurred. The change in the ratio of hexosamine to protein in the alpha-1 globulin in the diseases studied could be due to an increase of one of the components which migrates with the alpha-1 fraction which is considerably richer in hexosamine than the bulk of the protein in that fraction.

The electrophoretic method used in this study achieved good separation of the albumin and alpha-1 globulins, and the serum albumin was found to contain very small amounts of hexosamine. This has been noted in purified specimens for both hexose and hexosamine (5, 20). In the diseases studied, increases in the ratio of total serum hexosamine to total serum protein were noted (Table I), but electrophoretic studies showed significant increases in the ratio of hexosamine to protein in only one fraction. The observed changes in the alpha-1 globulin could not account for the increase in ratio of total serum hexosamine to total protein. The data for the electrophoretically separated serum pools revealed a fall in albumin in each case (Table II), which was quantitatively great enough to account for the bulk of the increase in ratio of total hexosamine to total protein, since the albumin contained so little of the total hexosamine.

Studies of the seromucoid have revealed similar changes in protein content in various diseases, but no change in the ratio of hexose to tyrosine in this fraction was found in several diseases (11, 12), although an increase in hexose/tyrosine was reported in rheumatoid arthritis, gout (7), and experimental scurvy (21). Our findings revealed a non-specific increase in the ratio of hexosamine to protein in the seromucoid fraction in disease. Much of the protein in the seromucoid migrates, at pH 8.6, as an alpha-1 globulin (2, 22). It is therefore of interest that the alpha-1 globulin alone among the electrophoretic serum protein fractions studied revealed an increase in the hexosamine to protein ratio with disease. When comparisons were made on individual sera or pools of sera it was apparent that the entire change in hexosamine content of the alpha-1 globulin could not be accounted for by the change in seromucoid hexosamine. It was not possible, with these methods, to determine if the entire change in hexosamine to protein ratio in the alpha-1 globulin could be accounted for by the change in hexosamine to protein ratio in the seromucoid.

Corticotropin has been found to produce an increase in the seromucoid in normal individuals (23). In patients with rheumatic fever, corticotropin lowered the initially elevated seromucoid level, but not entirely to normal, reaching a plateau and remaining somewhat elevated after other "acute phase reactants" were normal. When the corticotropin was discontinued, the seromucoid level fell the rest of the way to normal (24). Our findings in regard to the protein content of the seromucoid are similar. Those patients who had small elevations of the seromucoid protein level before therapy had an increase in the protein content of this fraction during therapy with anti-inflammatory hormones. In patients with rheumatic fever, who had markedly elevated levels before therapy, we also noted that hormones resulted in a fall of the protein content of the seromucoid toward, but not to, normal. It seemed that the direction of change with therapy depended on

the initial level, but the final level achieved was similar in all instances. In addition, the hexosamine content of the seromucoid fraction, was found to decrease during hormone administration in all instances. Thus the hexosamine decreased while the protein content of the seromucoid increased in those instances in which the protein rose with therapy. The ratio of hexosamine to protein fell toward normal in all.

These data seem to indicate that there are physiological differences among the components of the seromucoid fraction also. One or more components particularly rich in hexosamine may be increased by disease, while others containing proportionately less hexosamine may remain unchanged or undergo less of an increase. The hexosamine to protein ratio thereby increases during activity of the disease process. Suppressive hormone therapy apparently lowers the hexosamine to protein ratio by lowering the hexosamine-rich material selectively or predominantly. Since the protein level rises with hormone therapy if it is low initially, one component of this fraction, at least, must be increased by these agents. Presumably the latter component is not as rich in hexosamine as that lowered by therapy, since the total hexosamine and hexosamine to protein ratio decreases with hormone therapy.

The available information in the literature and that obtained by the methods used in this study seem to indicate differences in the significance of the various carbohydrate-containing fractions in the serum. It seems likely that further study of the physiology of individual components will be of interest.

SUMMARY

1. The total serum hexosamine rises in several diseases, leading to an increase in the ratio of total serum hexosamine to total serum protein.

2. Electrophoretic separation of serum proteins at pH 8.6 showed, in most of the diseases studied, a rise in the hexosamine associated with the alpha-1 globulin which was greater than the accompanying rise in protein content of the alpha-1 globulin, resulting in an increase in the ratio of hexosamine to protein. In the alpha-2, beta and gamma globulin fractions, parallel changes in hexosamine and protein occurred, with no significant changes in ratio.

3. The perchloric acid-soluble fraction, or seromucoid, also increases non-specifically with disease, but the hexosamine content was found to increase more than the protein, raising the ratio of hexosamine to protein in this fraction.

4. Suppression of inflammation with hormone therapy was found to decrease the hexosamine content of the alpha-1 globulin and seromucoid and lower the ratio of hexosamine to protein in those fractions.

5. These observations are interpreted as indicating that in the seromucoid and the alpha-1 globulins there seems to be an increase in at least one component which is richer in hexosamine than the rest of the fraction in a variety of illnesses, and that there is a fall in a hexosamine-rich component with anti-inflammatory hormone therapy.

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PULMONARY AND CIRCULATORY EFFECTS OF ACUTE PULMONARY VASCULAR ENGORGEMENT IN NORMAL SUBJECTS [1]

By STUART BONDURANT,[2] JOHN B. HICKAM, AND JOSEPH K. ISLEY

(From The Departments of Medicine and Radiology, Duke University School of Medicine, Durham, N. C.)

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Central and pulmonary vascular engorgement is a characteristic feature of congestive heart failure. It has been suggested many times that this vascular engorgement might be responsible for certain phenomena associated with congestive failure, such as stiffening of the lungs, dyspnea, and orthopnea. However, the simultaneous occurrence of pulmonary edema and other changes has made it difficult to single out the effects of simple vascular engorgement.

The present study was undertaken to investigate the pulmonary and circulatory effects of acute, reversible central and pulmonary vascular engorgement in normal man.

Central and pulmonary vascular engorgement was produced by rapid application of pressure over the surface of the body. Two methods were used to accomplish this: inflation of an aviator's "G suit," and submersion in water while breathing against atmospheric pressure.

The results indicate that vascular engorgement of the degree found in congestive heart failure can markedly stiffen the lungs. In addition, it was found that the production of acute central and pulmonary vascular engorgement in normal subjects by the present experimental means is quickly followed by changes which reduce the engorgement and which may represent an adaptive circulatory response. These changes could be modified by pre-treatment with drugs which alter vascular tone or by applying a painful stimulus.

METHODS

Subjects. All subjects studied in G suit experiments were male students 20 to 30 years of age. Three of the subjects studied during submersion were females, in the same age group. The subjects were indoctrinated by several dummy runs before measurements were made to minimize the effects of anxiety.

G Suit. The G suit [3] was a single chamber, balloon type garment which covered the feet, legs, and lower abdomen. The suit could be inflated to the desired pressure within 5 seconds by a standard Air Force G-valve. Suit pressure of 2 PSI (103 mm. Hg) was customarily used. The subjects were studied in the seated position before, during and after a 2-minute period of suit inflation.

Submersion. Seated subjects were studied before, during and after submersion in water to the neck and with the head just below the surface. At this depth, the mean pressure at hip level during total submersion was approximately 60 mm. Hg or 1.2 PSI.

Ventilatory measurements. Ventilation was measured with a wire screen flowmeter using a Statham differential strain gage. The amplified flow signal was either recorded directly or electrically integrated [4] to record respiratory volume change. During the G suit experiment, subjects breathed through a mouth piece almost directly into the flowmeter. Before and during submersion the mouth piece led to a 2.5 foot length of 1 inch I.D. corrugated tubing which in turn was attached to the flowmeter. Although this provided an undesirably large dead space, the same arrangement was used during both control and submersion studies, and the respiratory rates remained at all times within the usual normal limits. Tidal volumes with this arrangement were greater than normal and amounted to about 1000 ml. during both control and submersion periods.

Esophageal pressure was measured using an esophageal balloon as described by Mead and Whittenberger (1). The difference between esophageal pressure and mouth pressure was measured by means of a differential strain gage. This pressure, the "transpulmonary pressure," was recorded simultaneously with the flow and volume measurements.

From the recordings of flow, volume, and pressure, pulmonary compliance and resistance were measured as described by Mead and Whittenberger (1). Compliance is expressed as L. per cm. H_2O and resistance as cm. H_2O per L. per sec. at a flow of 0.5 L. per sec. In each

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[2] U. S. Public Health Service Post-Doctorate Research Fellow.

[3] Made by the David Clark Company, Inc., Worchester, Mass.

[4] Modified Technitrol Integrator.

instance, compliance was measured on five successive breaths and the mean value used. Thus, for example, the pulmonary compliance at 30 seconds after G suit inflation or submersion in water, listed below, actually represents the mean value of the five breaths nearest the 30-second mark. In like manner, the respiratory resistance values represent a mean of five determinations in each instance.

Inflation of the G suit caused a decrease in functional residual capacity amounting to about 500 ml. Consequently, it was necessary to determine whether this change alone would affect the measurement of compliance, although volume changes of this amount do not significantly alter compliance under normal conditions (*2*). In 3 subjects, the end-expiratory lung volume was voluntarily held at the control level during G suit inflation. This was done by maintaining a constant end-expiratory position on the tidal volume record. Compliance changes during G suit inflation were found on repeated trials to be unaffected by controlling the lung volume in this way.

The possibility arises that compliance measurements might be affected by a reflex change in esophageal tone induced by inflation of the G suit or submersion in water. These procedures caused a small immediate increase in the absolute level of intraesophageal pressure which was commensurate with the attendant decrease in resting lung volume and was ascribed to this cause. The new pressure level was stable. There was no increased tendency for intraesophageal pressure to fluctuate widely. On release of G suit pressure or emersion of the subject from water, the mean level of intraesophageal pressure decreased promptly again. The small pressure change which did occur was consistent, stable, and reversible. For this reason it appears unlikely that G suit inflation or immersion in water caused reflex changes in esophageal tone which could account for the measured changes in compliance.

Circulatory measurements. Brachial artery pressure was measured by way of an indwelling arterial needle. Central venous pressure was measured through a catheter (0.065 inch I.D.) in the superior vena cava. The fourth sterno-costal junction was taken as the reference point. Statham strain gages served as transducers for both measurements.

As a rough index of changes in intrathoracic blood volume during G suit inflation, radiographic density of the lungs was followed fluoroscopically and by roentgenograms, using standard exposure and development techniques. A series of control films was made at different depths of inspiration. Films made during G suit inflation were compared with control films showing the diaphragm at the same level.

As a further index of changes in intrathoracic blood volume, thoracic radioactivity was followed continuously during G suit inflation after an intravenous injection of 10 μc. of I^{131} albumin. Monitoring was accomplished by a sodium iodide, thallium activated scintillation crystal detector with the amplified signal recorded on a direct writing apparatus. The detector was shielded to monitor an area 5 inches in diameter at a distance of 6 inches. It was positioned over the right mid-lung field to exclude the heart and liver from the area monitored. Studies were performed within five minutes after injection when essentially all of the radioactive material remained in the blood. In order to determine whether this arrangement could reflect directional changes in intrathoracic blood volume, a trial was made in each subject of the effect of reducing intrathoracic blood volume by performing a Valsalva maneuver. In each case, this caused a definite

TABLE I

Pulmonary compliance, central venous pressure and arterial pressure during G suit inflation (2 psi)

Time after G suit inflation (*seconds*)		Pulmonary compliance *L./cm. H_2O*				Central venous pressure change *cm. H_2O*			Arterial pressure *mm. Hg*	
		Control	30	60	120	30	60	120	Control	30–120
Subject										
RS	1	.245	.167		.242					
TH	2	.182	.068	.136	.174	17.0	12.0			
CT	3	.328		.148	.202	39.5	32.2	25.4		
SB	4	.229	.092	.118	.145					
RN	5	.219	.066	.117	.127	23.0	16.0	13.0	122/70	150/100
CM	6	.212	.098	.111	.171	40.7	38.6	35.2		
KW	7	.281	.124	.130	.146	30.0	25.0	22.5		
ED	8	.214	.096	.122	.147	22.0	20.0	15.5	118/63	139/86
LB	9	.168	.076	.095	.103	21.7	17.7	13.0	96/70	120/80
ST	10	.220	.106	.133	.143	20.0	15.7	12.0	100/64	125/75
MM	11	.171	.121	.146	.150	27.0	23.0	18.0	110/70	120/75
WC	12	.128	.055	.075	.078	30.3	29.3	24.2	105/50	130/70
RS	13	.200	.125	.135	.145	18.5	14.1	10.4	95/56	100/68
CH	14	.209	.066	.079	.081	35.0	27.0	22.0	95/50	130/100
RK	15	.249	.044	.058	.064	23.4	18.9	15.5		
Average		.217	.093	.114	.141	26.8	22.3	18.9		
Std. Dev.		.048	.036	.027	.046	7.8	7.8	7.2		

TABLE II

Pulmonary compliance and respiratory resistance during submersion while breathing against atmospheric pressure

Depth of submersion	Pulmonary compliance L./cm. H_2O					Inspiratory resistance cm. H_2O/L./sec.	
	Control	Neck level		Head under		Control	Head under
Time after submersion seconds	Control	30	120	30	120	Control	120
Subject							
WD	.150			.093	.090		
GH	.185	.104	.118	.047	.093	0.9	0.9
FM	.138	.084	.095	.080	.102	1.4	1.8
JN	.168	.062	.080	.049	.104		
SB	.177	.120	.132	.051	.095	1.2	2.0
RF	.224	.122	.128	.076	.094	1.2	1.6
LB	.179	.133	.186	.061	.079		
LH	.125	.072	.072	.081	.144		
Average	.168	.100	.116	.077	.100		
Std. Dev.	.031	.042	.039	.027	.021		

decrease in radioactivity over the test area while intrathoracic pressure was increased. This result was taken to indicate the probability that changes in thoracic radioactivity which occurred during G suit inflation at least partially reflected changes in intrathoracic blood volume.

G suit inflation after pretreatment with hexamethonium. Intravenous injections of hexamethonium bromide were given to seated subjects to lower systolic pressure to approximately 70 mm. Hg. There was a wide variation in the dosage required to accomplish this. The mean dose (four subjects) was 225 mgm. Central venous pressure, arterial pressure and pulmonary compliance were measured during G suit inflation before and after administration of hexamethonium.

G suit inflation during norepinephrine infusion. The central venous catheter was used to allow alternate 5-second intervals of central venous pressure measurement and infusion of norepinephrine at a constant rate. The rate of infusion was adjusted to maintain the systolic blood pressure at approximately 170 mm. Hg. Central venous pressure, arterial pressure, pulmonary compliance and thoracic radioactivity were evaluated before and during G suit inflation both before and with norepinephrine infusion.

RESULTS

Changes during the first 30 seconds of G suit inflation (Table I)

The central venous pressure of each subject rose markedly upon inflation of the G suit. At 30 seconds, the mean increase in 13 subjects was 26.8 cm. H_2O. Pulmonary compliance decreased in every instance following G suit inflation (14 subjects). The mean control compliance was .217 L. per cm. H_2O. At 30 seconds after G suit inflation the group mean was .093 L. per cm. H_2O. Arterial pressure increased (8 subjects) by a group mean of 22 mm. systolic and 20 mm. diastolic at the 30-second point. The peak pressure, which occurred immediately after suit inflation, was slightly higher than this, but after a few seconds the pressure stabilized at a nearly constant level. There was a marked increase in radiographic pulmonary vascular density and in transverse cardiac diameter (4 subjects) as compared with control films showing the diaphragm at approximately the same level. All these changes were maximal within 15 seconds after G suit inflation. Thoracic radioactivity began a sharp increase with G suit inflation and reached a peak 15 to 30 seconds thereafter (5 subjects). There was no change in respiratory rate.

Changes during the first 30 seconds of submersion (Table II)

The effect of submersion was similar to that of G suit inflation. Pulmonary compliance decreased from a mean control value of .168 L. per cm. H_2O to .100 L. per cm. H_2O with 30 seconds' submersion to the level of the neck (7 subjects) and to .077 L. per cm. H_2O with 30 seconds' total submersion (8 subjects). Respiratory resistance was not greatly changed by submersion (4 subjects). Respiratory rate did not change with submersion.

Changes during prolonged G suit inflation

Further changes during the 2-minute period of G suit inflation are detailed in Table I. As G suit

pressure was maintained, the high central venous pressure began to decrease gradually. The mean increment above control level was 26.8 cm. H_2O at 30 seconds (13 subjects), 22.3 cm. H_2O at 60 seconds (13 subjects) and 18.9 cm. H_2O at 120 seconds (12 subjects). Correspondingly, pulmonary compliance, after the initial fall, began to increase and continued increasing throughout the last 1½ minutes of constant suit pressure. The mean compliance was .093 L. per cm. H_2O at 30 seconds (14 subjects), .114 L. per cm. H_2O at 60 seconds (14 subjects) and .141 L. per cm. H_2O at 120 seconds after G suit inflation (15 subjects). These progressive changes in central venous pressure and compliance were observed in each subject.

Following the initial increase in radiographic pulmonary density with G suit inflation, definite but incomplete clearing of the increment in density was observed by fluoroscopy and by films during the 2 minutes of continued G suit pressure (4 subjects).

Thoracic radioactivity, after the initial increase with G suit inflation, decreased throughout the remainder of the 2 minutes of continued suit pressure in 4 of 5 subjects. In the remaining subject, thoracic radioactivity showed the usual initial increase with suit inflation, but did not decrease during the remainder of the 2-minute period.

Arterial pressure and respiratory rate did not change during this prolonged interval.

The finding that radiographic pulmonary density and thoracic radioactivity began to decrease while G suit pressure was maintained indicates that the intrathoracic blood volume was also decreasing. This was associated with decreasing central venous pressure and increasing compliance of the lungs. These changes all began within 30 seconds after G suit inflation and the return toward control values continued during the remaining period of constant G suit pressure.

Changes during prolonged submersion (Table II)

The changes during submersion for 2 minutes were similar to those during prolonged G suit inflation. Following the initial decrease in pulmonary compliance with submersion, increasing compliance was observed. During submersion to the neck, compliance increased in 7 subjects from a mean of .100 L. per cm. H_2O after 30 seconds to a mean of .116 L. per cm. H_2O after 120 seconds. This change is statistically significant ($P < .05$). In 8 subjects during total submersion compliance increased from a mean of .077 L. per cm. H_2O after 30 seconds to a mean of .100 L. per cm. H_2O after 120 seconds ($P < .01$).

The effect on pulmonary compliance of submersion, like that of inflation of the G suit, is an

TABLE III

Pulmonary compliance, central venous pressure and arterial pressure during G suit inflation before and after hexamethonium bromide

Time after G suit inflation (seconds)	Pulmonary compliance L./cm. H_2O				Central venous pressure change cm. H_2O			Arterial pressure mm. Hg	
	Control	30	60	120	30	60	120	Control	30–120
Before hexamethonium									
Subject									
ST	.164	.050	.054	.072	29	27	23	100/64	125/75
WC	.128	.055	.075	.078	30	29	24	105/50	130/70
MM	.190	.121	.146	.152	27	23	18	110/70	120/75
LB	.234	.079	.076	.094	28	26	22	96/70	120/80
Average	.179	.076	.088	.098	28.6	26.3	21.8	103/63	124/75
After hexamethonium									
ST	.229	.084	.096	.102	17	14	13.5	60/40	
WC	.137	.088	.072	.087	19	20	17	90/50	120/80
MM	.176	.105	.092	.108	14.5	14.5	13	68/40	138/80
LB	.236	.058	.065	.074	20	17	15	76/50	110/80
Average	.194	.084	.081	.098	17.5	16.4	14.6	73/45	123/80

TABLE IV

Pulmonary compliance, central venous pressure and arterial pressure during G suit inflation before and during norepinephrine infusion

Time after G suit inflation (seconds)	Pulmonary compliance L./cm. H_2O				Central venous pressure cm. H_2O				Arterial pressure mm. Hg	
	Control	30	60	120	Control	30	60	120	Control	30–120
Before norepinephrine infusion										
Subject										
RS	.200	.125	.135	.145	2.0	20.5	16.1	12.4	95/56	100/68
RN	.184	.083	.090	.096	1.5	29.5	25.5	22.5	122/70	150/100
CH	.209	.066	.079	.081	1.5	36.5	28.5	26.5	95/50	130/100
ED	.214	.096	.122	.147	0.5	22.5	20.5	16.0	118/63	139/86
LB	.216	.076	.095	.103	1.0	23.2	19.0	14.5	88/50	126/73
Average	.204	.089	.104	.114	1.3	25.9	21.9	18.3	103/58	129/86
Std. Dev.	.013	.022	.023	.028	0.1	6.4	5.0	6.1		
During norepinephrine infusion										
RS	.111	.075	.080	.081	8.5	35.2	34.8	34.8	165/82	177/105
RN	.147	.064	.063	.061	4.0	34.0	32.5	32.0	183/78	191/99
CH	.167	.043	.046	.050	5.0	44.4	44.6	43.5	150/84	157/100
ED	.176	.082	.082	.086	6.5	46.5	48.5	48.5	192/82	205/110
LB	.209	.056	.060	.060	8.5	40.5	42.0	45.0	160/85	180/100
Average	.162	.064	.066	.068	6.5	40.1	40.5	40.8	170/82	182/103
Std. Dev.	.036	.015	.015	.015	2.0	5.5	6.7	7.0		

initial sharp decrease with subsequent incomplete return toward control values during continued submersion.

Changes during G suit inflation after hexamethonium injection (Table III)

G suit inflation after hexamethonium injection produced in 4 subjects a less marked increase in central venous pressure (mean 17.5 cm. H_2O) than it produced during control observations in the same subjects prior to hexamethonium injection (28.6 cm. H_2O). During continued G suit inflation a progressive fall in central venous pressure was again apparent, but it was less marked than that which occurred during the control observations.

Pre-treatment with hexamethonium did not alter the pulmonary compliance changes associated with G suit pressure.

Changes during norepinephrine infusion (Table IV)

In 5 seated subjects norepinephrine infusion consistently produced an increase in central venous pressure and a decrease in compliance. There was no detectable change in thoracic radioactivity during norepinephrine infusion.

Changes during G suit inflation with norepinephrine infusion (Table IV)

G suit inflation during norepinephrine infusion resulted in a marked immediate increase in central venous pressure and a simultaneous decrease in pulmonary compliance. During the 2-minute period of constant G suit pressure, there was no significant further change in either of these values.

Thoracic radioactivity increased markedly with G suit inflation during norepinephrine infusion (3 subjects). During continued G suit pressure, thoracic radioactivity either remained constant or continued to increase slightly.

Immersion of arm in ice water during G suit inflation

The effect of a strong autonomic stimulus was examined in 2 subjects by immersing one arm deeply in agitated ice water for 2 minutes. As indicated in Table V, this procedure alone caused a definite fall in compliance and an elevation in central venous pressure. Table VI shows the effect of immersing the arm in ice water coincident with G suit inflation. As with norepinephrine infusion, the initial changes in compliance and venous pressure tend to be maintained during the

TABLE V

Effect of immersion of the arm in ice water on pulmonary compliance and central venous pressure

Time after immersion (*seconds*)	Pulmonary compliance *L./cm. H_2O*		Central venous pressure *cm. H_2O*	
	Control	120	Control	120
Subject				
PK	.170	.098	1.0	3.0
MM	.200	.135	1.5	6.0

entire 2-minute period instead of returning toward control values.

Release of G suit pressure and emersion from water

All measured values returned to control range within 10 seconds after release of G suit pressure or emersion from water. Invariably arterial pressure and central venous pressure fell below control levels for 3 to 5 seconds after release of G suit pressure.

DISCUSSION

Inflation of the G suit caused marked central and pulmonary vascular engorgement, as evidenced by an increase in central venous pressure, in radiographic density of the lungs, and in thoracic radioactivity after injection of I^{131} labeled albumin. Pulmonary engorgement was always accompanied by a large decrease in compliance. Upon release of suit pressure, these changes all disappeared promptly. The compliance change produced by G suit inflation was quite comparable in magnitude to that which is found in congestive heart failure (3).

The present evidence indicates that simple pulmonary vascular engorgement can produce a marked decrease in compliance, but the manner in which engorgement acts to produce this effect is not clear. It has been suggested that the lungs may be stiffened in some way by an increase in pulmonary intravascular pressures (3–8).

This factor would certainly be operative under the present conditions. It has been reported that inflation of a G suit causes an elevation of intravascular pressure throughout the pulmonary circulation comparable to the increase produced in the central venous pressure (9). However, G suit inflation also increases intrathoracic blood volume, and the present study does not exclude this factor as a determinant of compliance changes.

Similar changes in compliance have been produced by increasing the pulmonary vascular pressure in experimental animals (4, 6, 7), and rapid intravenous infusions have been reported to produce a compliance fall in normal human subjects (10). These results also suggest that simple pulmonary vascular engorgement can produce a decrease in compliance.

The mechanism of the decreased compliance in congestive heart failure appears to be somewhat more complicated. Brown, Fry, and Ebert (3) found that pulmonary compliance was decreased in patients with congestive failure, and the extent

TABLE VI

Effect of immersion of the arm in ice water on the change in pulmonary compliance and central venous pressure during G suit inflation

Time after G suit inflation (*seconds*)	Pulmonary compliance *L./cm. H_2O*				Central venous pressure *cm. H_2O*			
	Control	30	60	120	Control	30	60	120
Before immersion of arm in ice water								
Subject								
PK	.178	.055	.071	.076	1.0	22	15	11
MM	.200	.107	.120	.126	1.5	29	27	24
Average	.189	.081	.095	.100	1.2	25.5	21	17.5
During immersion of arm in ice water								
PK	.168	.069	.079	.070	1.0	33	24	28
MM	.210	.079	.083	.085	1.5	22	30	26
Average	.189	.074	.081	.076	1.2	27	27	27

of the change appeared to be related to the severity of the failure. There was a good correlation between decrease in compliance and reduction in vital capacity. Examinations have been made of the effect on compliance of elevating pulmonary vascular pressures by exercise in patients with mitral stenosis (8, 11). This often causes a decrease in compliance, but it may fail to do so in cases where compliance is already much reduced at rest. In such cases, the reduction in compliance may depend on the fibrotic pulmonary changes which are known to occur in severe mitral stenosis. It is probable that pulmonary edema also contributes to the reduced compliance of patients with congestive failure. Pulmonary edema produced by ANTU will lower compliance in the dog (12). In general, it appears that simple pulmonary vascular engorgement can reduce compliance to the extent seen in congestive heart failure, but that the compliance reduction in this disorder may depend on other factors as well.

Although most of the subjects were trained observers, only one complained spontaneously of dyspnea during G suit inflation. Two others described upon questioning a vague sense of shortness of breath during the experiments. The youth and vigor of the subjects as well as the acute nature of the experiments render difficult the interpretation of this subjective sensation. It is evident that the healthy young adult tolerates very low pulmonary compliance for short periods without experiencing dyspnea at rest.

On inflating the G suit and maintaining its pressure, there was at first an abrupt increase in central and pulmonary vascular engorgement, and then a gradual decrease, as indicated by a progressive decline in central venous pressure, in radiographic density of the lungs and in thoracic radioactivity, and by a simultaneous increase in compliance. Similar compliance changes occurred during submersion. These changes suggest that the initial abrupt increase in intrathoracic blood volume is being reduced by redistribution of blood to the periphery. The combination of a gain in peripheral blood volume occurring conjointly with a fall in central venous pressure might be produced by an overall fall in peripheral vascular tone, particularly venous tone. It might also be caused by pooling of blood in vascular regions which are under less than the mean externally applied pressure and which can accept additional blood from the arterial side until the local venous pressure rises sufficiently to open venous valves and re-establish a return flow into the general venous system. The first of these mechanisms would represent a vasodepressor response, the second a passive pooling. To investigate this subject further, trials were made of the effect of altering venomotor tone by appropriate drugs before G suit inflation.

Partial ganglionic blockade with hexamethonium greatly reduced the venous pressure rise after G suit inflation, possibly because of a decrease in "resting" venomotor tone, but the effect on compliance was unchanged. This response does not differentiate between the two mechanisms.

Pre-treatment with norepinephrine abolished the evidence of redistribution of blood from the central region back into the periphery. It is conceivable that this blocking effect might result either because the direct action of norepinephrine on vessels prevented the occurrence of a vasodepressor response or because overall venous tone was so increased that no significant passive pooling could occur. Central venous pressure was increased by norepinephrine alone and was very greatly increased by subsequent inflation of the G suit.

The existence of venomotor reflexes which are capable of producing a considerable increase in the central venous pressure is well established (13, 14). Whether the present redistribution phenomenon represents an actual venodepressor response or passive pooling, it is apparent that the extent of the initial rise in venous pressure and the subsequent course of events depend very closely on venous tone. It is still not clear whether a decrease in venous tone occurs during the period of G suit inflation and contributes to the redistribution of blood.

The ice water experiments indicate that the nervous system can mediate changes in venous tone in normal subjects which are associated not only with an increase in central venous pressure but also with a considerable fall in compliance. By analogy with the other experiments in which central venous pressure is elevated, it appears likely that this decrease in compliance reflects some degree of pulmonary vascular engorgement resulting from a transfer of blood from the systemic to the pulmonary circulation. When cen-

tral and pulmonary vascular engorgement are already present, as after G suit inflation, the nervous system can mediate an increase in venous tone which is able to sustain very high pressures and which apparently maintains pulmonary engorgement by resisting transfer of blood from the pulmonary to the systemic circulation. The results obtained with ice water closely simulate those obtained during norepinephrine infusion.

SUMMARY

1. Brief, acute central and pulmonary vascular engorgement was induced in normal subjects by application of pressure to the body surface. This was done either by inflation of an aviator's G suit to 2 PSI or by submersion in water while breathing against atmospheric pressure. G suit inflation produced in 15 subjects an increase in central venous pressure of 26.8 (S.D. 7.8) cm. H_2O and a fall in compliance from the control value of .217 ± .048 L. per cm. H_2O to .093 ± .036 L. per cm. H_2O. A similar compliance fall occurred with submersion.

2. On maintaining G suit pressure or submersion for a 2-minute period, central venous pressure and compliance showed a progressive, but incomplete, return toward control levels. Infusion of norepinephrine or immersion of an arm in ice water during this time prevented the return of venous pressure and compliance toward control levels.

3. Administration of norepinephrine or immersion of an arm in ice water without suit inflation caused a moderate increase in central venous pressure and a moderate decrease in compliance.

4. When the G suit was inflated during norepinephrine infusion, the resultant elevation of central venous pressure and the drop in compliance were greater than in untreated subjects. Pretreatment with hexamethonium diminished the rise in central venous pressure which occurred with suit inflation, but did not affect the compliance changes.

5. It is suggested that the compliance changes induced by these procedures depend, at least partially, on varying degrees of pulmonary vascular engorgement and that this, in turn, is partially dependent, under the present experimental conditions, on peripheral venous tone.

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THE EFFECT OF INTRAVENOUS CORTISOL INJECTIONS ON THE PLASMA CORTISOL CONCENTRATION IN MAN [1]

By JAMES SCHEUER [2] AND PHILIP K. BONDY

(From the Department of Medicine, Yale University School of Medicine, New Haven, Conn.)

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Previous workers have shown (1–7) that 17-hydroxycorticosteroids disappear rapidly from the plasma after cortisol (hydrocortisone) is injected intravenously. The disappearance rates have not, however, been studied in detail nor have analytical methods been used which would distinguish cortisol from its reduced derivatives. In the present experiments disappearance curves of cortisol have been determined, both after sudden massive injections, and during slow infusions at rates which permitted the establishment of equilibrium at physiological plasma cortisol concentrations. Steroid determinations have been performed by methods which separate cortisol from all other steroids known to be present in human plasma.

MATERIALS AND METHODS

All subjects were healthy male medical students between the ages of 21 and 25 years, without evidence of serious disease or history of hepatic, renal or endocrinological abnormality. Studies were started between 8:30 and 9:30 a.m. The subjects were ambulatory but otherwise inactive. They were on normal diets throughout the experiment and all understood the nature of the procedure. Five subjects were used in each part and those who participated in Part I were not used in Part II.

Heparinized blood samples were centrifuged immediately upon withdrawal and except in a few cases were extracted the same day. On occasion it was necessary to freeze the plasma for a few days prior to extraction. The plasma samples were analyzed by the method of Bondy, Abelson, Scheuer, Tseu, and Upton (8, 9), a brief description of which follows.

Appropriate duplicate quantities of plasma were extracted with chloroform and the extracts chromatographed in the Bush toluene-75 per cent methanol paper chromatography system. Cortisol spots were eluted with ethyl alcohol and the quantity of steroid measured by potassium butoxide fluorometry (8). Small amounts of cortisol-4-C^{14} were added to the original plasma sample and the radioactive recovery used to correct for losses during the procedure. The calculated standard deviation between duplicate samples with this method is ± 7 per cent, and recovery of added cortisol is 100 ± 14 per cent.

EXPERIMENTAL PROCEDURE

Part I: A control blood specimen was drawn and then 100 mg. of cortisol (Upjohn) in 150 ml. of 6.7 per cent alcohol and 5 per cent glucose in water were injected over a 5-minute period through the same needle. Blood samples were taken at 15, 60, 150, 300, and 420 minutes from a vein in the opposite arm. In two subjects samples were taken every 15 minutes for the first hour and every 20 minutes for the second hour. The total amount of blood drawn was 320 ml.

Part II: After an initial blood sample had been drawn, an infusion of cortisol in 500 ml. of 5 per cent glucose (Merck) was injected intravenously through a constant infusion pump at approximately 300 μg. per minute. Thereafter blood was withdrawn from the opposite arm at 60, 165, 180, 300, and 480 minutes. After withdrawal of the 180-minute sample the infusion was stopped and the solution run through the pump and needle into a 100-ml. volumetric flask for 10 minutes. The flask was then filled to volume with distilled water. An appropriate aliquot was extracted with chloroform three times. The chloroform was evaporated and the residue dissolved in 6 ml. absolute ethyl alcohol and read on a Beckman Model DU spectrophotometer at a wave length of 242 mμ to determine accurately the amount of cortisol infused over a 10-minute period. The total amount of blood drawn was 360 ml.

Urine analyses were not performed because the portion of injected cortisol excreted unchanged by the kidney is very small (5).

CALCULATIONS

Part I: At the time of the first post-infusion sample it was assumed that the adrenal gland was no longer secreting cortisol and that the amount of endogenous cortisol present was relatively very small so that the plasma level was almost totally due to exogenous cortisol. If the disappearance of the steroid from the plasma is proportional to the concentration (2), it is represented by the equation:

$$-\frac{dc}{dt} = Kc \qquad (1)$$

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where c is the concentration in the theoretical volume of distribution, v. It is assumed that c is constant throughout v (see below). By integrating:

$$c = C_0 e^{-kt} \tag{2}$$

where C_0 is the concentration at time zero.

Part II: The same assumptions were made regarding the endogenous cortisol as in Part I. Since the cortisol was being added to the system at a constant rate, the change of concentration assuming no loss would be:

$$\frac{dc}{dt} = \frac{r}{v} \tag{3}$$

where r is rate of infusion. When the rate of disappearance of the steroid is taken into account (Equation 1) net increase in concentration may be represented as:

$$\frac{dc}{dt} = \frac{r}{v} - kc \tag{4}$$

If the concentration of cortisol was zero at time zero, then:

$$c = \frac{r}{kv}(1 - e^{-kt}) \tag{5}$$

When $t = \infty$, c reaches an asymptote:

$$c = \frac{r}{kv} \tag{6}$$

r is a known constant and the values for c are known at different times. Substituting these values in Equation 5, v and k, can be derived. The descending curve is analyzed by Equation 2.

The elimination of endogenous cortisol has been calculated in Parts I and II from Equation 2, assuming that disappearance of endogenous hormone proceeded at the same rate as exogenous cortisol when at equilibrium (7). By subtracting the calculated endogenous curves from the total, one obtains an approximation of the exogenous curves (Figures 1, 2, and 3).

RESULTS

Table I represents the levels of cortisol obtained after the acute injection of this substance. The concentrations differ widely about the mean, but each falls on a smooth curve. A graph of the

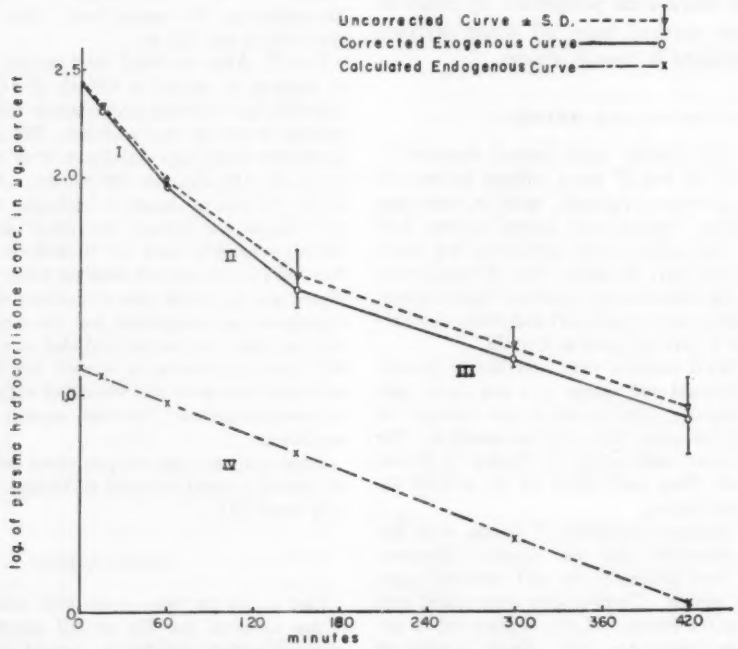

FIG. 1. MEAN CONCENTRATION OF PLASMA CORTISOL AFTER THE RAPID INJECTION OF 100 MG. OF CORTISOL

The equations for the various curves are as follows:

I. $c = 264\, e^{-0.0189t}$ $\quad t_{\frac{1}{2}} = 37$ minutes

II. $c = 88.1\, e^{-0.0120\,t}$ $\quad t_{\frac{1}{2}} = 58$ minutes

III. $c = 29.5\, e^{-.00514\,t}$ $\quad t_{\frac{1}{2}} = 138$ minutes

IV. $c = 13.1\, e^{-0.00514\,t}$

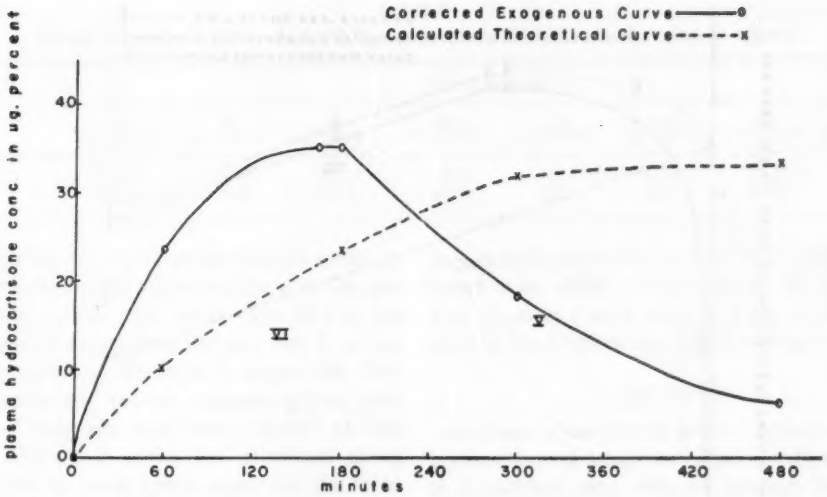

FIG. 2. MEAN CONCENTRATION OF PLASMA CORTISOL DURING CONTINUOUS INTRAVENOUS INJECTION OF CORTISOL AT A RATE OF 301 μg. PER MINUTE (SOLID LINE) AND THE CURVE WHICH WOULD HAVE BEEN OBTAINED IF THE RATE CONSTANT AND VOLUME OF DISTRIBUTION HAD BEEN CONSTANT THROUGHOUT THE EXPERIMENT

The infusion was stopped at 180 minutes. The equations for the curves are as follows:

$$\text{V.}\quad c = 35.2\, e^{-0.00568\, t} \qquad t_{\frac{1}{2}} = 124 \text{ minutes}$$

$$\text{VI.}\quad c = \frac{301}{1550\ (.00568)}\ (1 - e^{-0.00568\, t})$$

mean values is shown in Figure 1. During the first 150 minutes the rate of disappearance from the plasma decelerates fairly evenly so that the logarithm of the concentration forms a concave curve when plotted against time. This curve can be represented by two arbitrary straight lines which approximate the average rates of disappearance during those periods. Thus, during the first 60 minutes there is a rapid disappearance from the plasma at an average rate of 1.84 per cent per minute. Over the next 90 minutes the disappearance averages 1.20 per cent per minute. For the next 230 minutes the disappearance rate remains constant at 0.51 per cent per minute with a mean half-life of 138 minutes and a range among our subjects of 58 to 161 minutes.

Frequent samples were taken during the first 150 minutes in two subjects. The results are shown in Table II. Because of the very high levels of exogenous steroid during this early part of the curve, the endogenous levels represent a negligible portion of the plasma concentration and may be ignored. During this time there is a rapid fall, especially in subject JS where the level seems to fall to low values and then rebound upward to approach the mean curve. This early fall is much larger than the experimental error, since the standard deviation of the analytical method was only ± 8.5 per cent. The number of subjects is too small for general conclusions; however, the data demonstrate the rapidity of fall and the variability of the rate of disappearance immediately after a single, large injection of cortisol.

If one assumes that the plasma volume is 4 per cent of body weight, in these experiments 94.0 per cent of the administered dose had disappeared from the plasma after 15 minutes, 97.4 per cent at the end of 60 minutes, and 99.1 per cent after 150 minutes. In these instances the plasma cortisol level fell below the initial endogenous concentration 300 to 420 minutes after the steroid injection.

Table III represents the results obtained with the constant infusion in Part II. Here, as in Part I, there is a wide individual variation from the mean. The values at 165 minutes and 180 minutes are so similar for any given subject that it

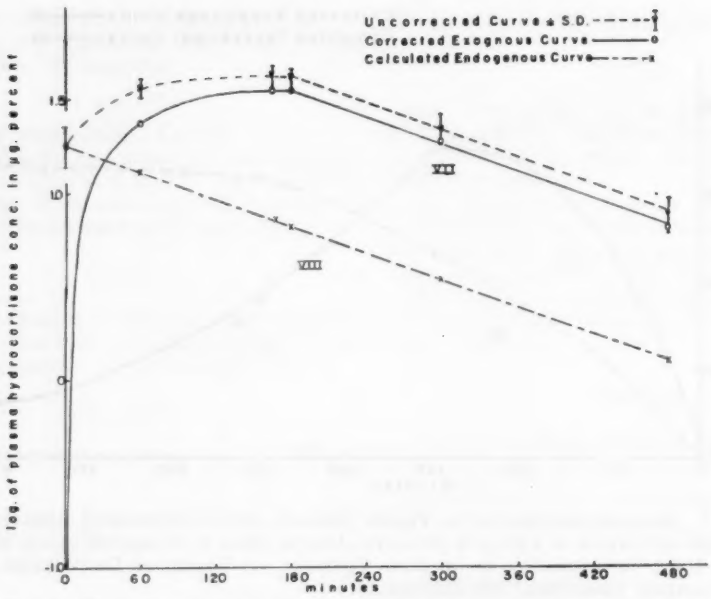

FIG. 3. MEAN CONCENTRATION OF PLASMA CORTISOL DURING CONTINUOUS INTRAVENOUS INJECTION OF CORTISOL AT A RATE OF 301 μg. PER MINUTE

Curve VII is the same as curve V, Figure 2, with the ordinate expressed as the logarithm of plasma cortisol concentration. The equation for curve VIII is as follows:

$$\text{VIII.}\quad c = 17.5\, e^{-0.00568\, t}$$

has been thought valid to average them and consider that the curve has reached its asymptote during this period. The equilibrium concentrations range from 25.8 to 66.9 μg. per cent. During the infusion the rate of disappearance of cortisol decreases constantly, leveling off during the final 15 minutes toward a half-life of 172 minutes. During this 15-minute period the theoretical volume of distribution (v in Equation 5) averages 116 per cent of body weight. The value of v may also be calculated from Equation 6, using the k value obtained from the falling limb of the curve, and c

TABLE I

Plasma cortisol levels after a single intravenous injection of cortisol—Part I

Subject	Weight kg.	Time in minutes					
		0 μg./100 ml.	15 μg./100 ml.	60 μg./100 ml.	150 μg./100 ml.	300 μg./100 ml.	420 μg./100 ml.
CH	92.9	10.0	140.0	84.0	14.5	2.5	8.2
AB	75.0	9.4	205.0	73.8	31.1	15.6	4.0
JC	71.8	4.5	179.0	63.0	37.5	19.5	12.0
JS	61.3	22.5	227.0	70.9	49.5	25.0	10.0
AH	72.8	18.9	314.0	197.0	39.0	21.0	8.7
Mean±SD	74.8	13.1±3.2	213.0±9.4	97.7±6.5	35.5±10.6	16.7±3.5	8.6±3.9
Calculated mean endogenous levels		13.1	12.1	9.6	6.0	2.8	1.5
Calculated mean exogenous levels (corrected for endogenous decay)		0	201.0	88.1	29.5	13.9	7.1

TABLE II

Plasma cortisol levels during the first 150 minutes after a single intravenous injection—Part I

Subject	Time in minutes							
	15 μg./100 ml.	30 μg./100 ml.	45 μg./100 ml.	60 μg./100 ml.	80 μg./100 ml.	100 μg./100 ml.	120 μg./100 ml.	150 μg./100 ml.
JS	227.0	136.0	42.2	70.9	42.2	83.3	55.5	49.5
AH	314.0	219.0	188.0	197.0	136.8	82.1	56.0	39.0

obtained during the last 15 minutes of the infusion. By this calculation the mean volume v is 186 per cent of body weight. The lower value of v in the first calculation arises from the fact that k on the ascending portion of the curve is larger than during the descending portion, suggesting that true equilibrium may not have been reached at 180 minutes. Figure 2 shows the calculated mean curve plotted on linear graph paper and the theoretical curve one would obtain if v and k were constant throughout and equal to the 180-minute values. Figure 3 shows the mean curve on semilogarithmic paper. In Part II, as in Part I, after equilibrium has been reached cortisol disappears from the blood at a rate of 0.57 per cent per minute with a half-life of 124 minutes and an individual range of 93.6 to 141 minutes. During the infusion, at 60 minutes 96.7 per cent, and at 180 minutes 97.9 per cent, of the administered cortisol had disappeared from the plasma.

Pre-infusion levels in all subjects in Part II, except TT, are above the normal range. At a later date samples were drawn from these subjects and found to be within normal range. At the end of Part II, as in Part I, there is a dip in the plasma cortisol level below the initial endogenous level.

DISCUSSION

In these experiments, after a period of equilibration cortisol disappears from the blood stream at a constant rate, with an average half-life of about 130 minutes. This confirms the observation by Eik-Nes, Sandberg, Nelson, Tyler, and Samuels (1) that the elevated blood 17-hydroxycorticosteroids induced by ACTH decreased at a rate proportional to the concentration. Similarly, Brown, Willardson, Samuels, and Tyler (3) gave intravenous cortisol over a 30-minute period and found that after 60 minutes the plasma 17-hydroxycorticosteroid concentration fell exponentially. Eik-Nes, Sandberg, Tyler, and Samuels (2) published a mean curve from which a half-life of about 120 minutes can be calculated. Done, Ely, Olsen, and Kelley (4) found that the half-life of 17-hydroxy-

TABLE III

Plasma cortisol levels during and after constant infusion of cortisol—Part II

Subject	Weight kg.	Inf. rate μg./min.	Time in minutes					
			0 μg./100 ml.	60 μg./100 ml.	165 μg./100 ml.	180 μg./100 ml.	300 μg./100 ml.	480 μg./100 ml.
JK	74.0	327	22.9	62.2	69.5	64.3	33.7	15.4
GE	77.6	371	21.6	37.2	38.8	37.8	18.4	4.3
RW	77.3	231	21.0	33.3	45.0	49.0	20.8	7.9
AH	88.7	378	15.0	24.0	25.8*	25.8	20.0	4.0
TT	95.5	227	7.2	23.6	36.2	28.8	15.2	5.5
Mean±SD	83.0	301	17.5±4.2	36.1±4.2	43.1±6.3	41.1±4.5	21.6±3.8	7.4±1.6
Calculated mean endogenous levels			17.5	12.6	7.0	6.5	3.3	1.2
Calculated mean exogenous levels (corrected for endogenous decay)			0	23.5	35.2†		18.3	6.2

* Because of error in analysis of the 165-minute sam ple there was no value obtained. The 180-minute value is used in order to preserve the mode of the average curve.

† The mean of the 165- and 180-minute values.

corticosteroids after intravenous injection of cortisol in normal subjects ranged from 60 to 133 minutes, with an average of 90 minutes. Sayers and his associates (5) observed disappearance of cortisol at a rate proportional to concentration, with half-lives of 78 to 138 minutes in one normal and two Addisonian patients. Peterson, Wyngaarden, Guerra, Brodie, and Bunim (6) found a half-life in 20 normal subjects of 114 ± 6.5 minutes, with a range of 90 to 130 minutes. This constant rate is due mainly to the activity of the liver, since Tomizawa, Narahara, Gibbons, and Williams (10) showed that hepatectomized rats did not metabolize cortisol, and Brown, Willardson, Samuels, and Tyler (3) found that in patients with liver disease the disappearance rate of 17-hydroxycorticosteroids was slowed proportional to the retention of bromosulfaphthalein. Peterson, Wyngaarden, Guerra, Brodie, and Bunim (6) also found increased half-lives in all their patients with liver disease.

The rapid initial removal of cortisol from the blood stream, described by others (3, 7), has been confirmed in the present experiments. Thus, Brown, Willardson, Samuels, and Tyler (3) found less than 5 per cent free 17-hydroxycorticosteroids in the plasma 60 minutes after intravenous administration, and Hellman and his associates (7) found only 13 per cent of C^{14}-labeled cortisol present in the plasma 15 minutes after infusion. In most of the previous experiments, the analytical methods used were somewhat nonspecific, since dihydrocortisol and tetrahydrocortisol would have been included in the "17-hydroxycorticosteroid" fraction if present in free form. Apparently negligible amounts of these substances are present, however, since the results obtained with a highly specific analytical technique in the present experiments are similar to those obtained with somewhat less specific methods.

In all of the previous studies, plasma concentrations were determined only 60 minutes or more after the steroid had been injected. If the data presented by Eik-Nes, Sandberg, Tyler, and Samuels (2) for individual curves are plotted on semi-logarithmic paper, the concentration in each case falls more rapidly between the first and second hour than during the remainder of the time.

In the present experiments the disappearance curve was not exponential for the first 150 minutes after the single intravenous injection of cortisol, but showed a steadily decreasing value for k. During the constant injection experiments also either k decreased or v increased, or both, until equilibrium was reached. This suggests that cortisol leaves the plasma more rapidly early in the experiment than it does after several hours. If the late exponential disappearance rate is constant during the early part of the experiments, which seems a reasonable assumption, then the accelerated rate observed in the initial hours, which accounts for about 48 per cent of the total disappearance as estimated from the areas under the curves, must reflect the presence of additional disposal systems besides the one which controls the rate of disappearance at equilibrium. Since accelerated cortisol disposal occurred early during the constant infusion experiments, the initial rapid rate of disappearance cannot be merely the result of elevated plasma cortisol concentration, or of delay in mixing throughout the volume of distribution. The loss of steroid directly by renal excretion can be ignored since the quantities of unmetabolized cortisol entering the urine are negligible (5). The temporary rapid disappearance rates early in both experiments therefore presumably represent the rapid entry of cortisol into body compartments other than the plasma (*i.e.*, a period of "equilibration" [10]). Since extracellular water contains no more cortisol than the plasma (11–16), the steroid must enter the cells. Levin, Daughaday, and Bremer have suggested that this equilibration is in part a result of binding of cortisol to tissue proteins (12), since many proteins can form nondialyzable complexes with steroids (13). In any case, the present data show that calculation of a single theoretical volume of distribution, v, is unwarranted since it is based on the assumption that the concentration of cortisol is the same throughout the entire volume of distribution as it is in the plasma. Since v proves to be larger than the total body volume both in these experiments and in those of others (6, 10), the concentration of cortisol in sites of binding outside of the plasma appears to be much higher than in the plasma.

The low values for cortisol found at the end of the experiment may be due to the suppression of ACTH by exogenous cortisol with a lag period after most of the exogenous cortisol has been

metabolized, leaving an adrenal that has had no stimulation for a few hours.

There must be many unrecognized variables which act upon the distribution and disappearance of cortisol to account for the wide range of individual values. Pre-injection steroid concentrations were frequently elevated in subjects later proved to be normal, suggesting that the test situation itself may alter in some way either the rate of endogenous secretion or rate of disposal of the hormone.

SUMMARY

1. One hundred mg. of cortisol were injected intravenously in 5 minutes into healthy subjects and the disappearance of the steroid from the plasma was followed. The rate of removal, which was very rapid at first, gradually decreased to about one-third of the original rate at 150 minutes, after which it remained constant with a mean half-life for plasma cortisol of 138 minutes.

2. In a second set of experiments, a constant intravenous infusion of approximately 300 μg. per minute was given. At 180 minutes equilibrium was reached when the amount leaving the plasma equalled the amount entering. The post-infusion curve was plotted and a mean half-life of 124 minutes was found. The volume of distribution of cortisol when the plasma concentration was at equilibrium was greater than the body weight, indicating that this "volume of distribution" has no real meaning.

3. Plasma cortisol concentrations a few hours after the infusion were lower than those found prior to the injection.

4. The results suggest that cortisol may leave the plasma in two general ways: via a reversible system in which the steroid is bound in high concentrations to tissue proteins; and an irreversible system whereby cortisol is permanently removed from the plasma. The early part of the disappearance curve is greatly influenced by the reversible binding of the steroid, whereas the later portion of the curve reflects a first-order metabolic process, presumably occurring chiefly in the liver.

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THE EFFECT OF GLUCAGON ON THE BLOOD GLUCOSE LEVEL AND THE CLINICAL STATE IN THE PRESENCE OF MARKED INSULIN HYPOGLYCEMIA [1, 2]

By JEROME L. SCHULMAN AND STANLEY E. GREBEN

(From the Department of Psychiatry of The Johns Hopkins Hospital, Baltimore, Md.)

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The increasing evidence that glucagon is a naturally occurring hormone has led to considerable interest and speculation as to its possible metabolic roles. The possibility of obtaining glucagon in increasingly pure preparations and the final obtainment of a crystalline preparation (1) have added impetus to this interest.

The presence of a hyperglycemic contaminant was one of the suggestions advanced to explain the initial rise in the blood sugar level which followed the administration of some preparations of insulin. There is general agreement that glucagon can cause at least a temporary rise in the blood sugar level in the presence of insulin. However, considerable interest still centers around the question of the relative effects of insulin and glucagon on the blood sugar level (2–5).

It was believed that there would be some advantage in exploring the effect of glucagon in patients who had received massive doses of insulin. This situation obtains in the insulin coma treatment of psychiatric patients. It was also considered that glucagon could serve as a useful means of terminating insulin coma. The present report deals with these questions.

MATERIALS AND METHODS

The patients used for this study were all hospitalized at The Henry Phipps Psychiatric Clinic and were diagnosed as exhibiting schizophrenic reactions. There were 11 patients, of whom 6 were women and 5 were men. They ranged in age from 16 to 48 years with an average age of 28 years. They were all in good physical health.

All of these patients were receiving insulin coma therapy as part of the therapeutic regimen for their psychiatric illness. A commercial preparation of regular insulin was employed. The dose of insulin varied from 60 to 2000 units with an average dose of 593 units for each treatment. On each treatment day the patient, in a fasting state, received crystalline insulin intramuscularly at 7:00 A.M. Typically, the patient entered coma at about 9:00 A.M. and the coma was terminated at about 10:30 A.M. At the time of termination the patient was almost invariably in third or fourth stage coma according to the criteria of Himwich (6). The usual course of treatments consisted of about 60 comas although there was some variation and glucagon was not employed for the termination of each coma.

Insulin comas were terminated with glucagon in 141 instances. Of this total glucagon was administered intravenously 97 times, intramuscularly 22 times and subcutaneously on 22 occasions. Following 61 of these terminations of coma the response to glucagon was followed with blood sugar determinations. Blood was withdrawn from an antecubital vein several minutes before the administration of the glucagon and at 5, 10, 15, 20, 30, 40, and 50 minutes following the administration of the glucagon. A separate venipuncture was performed for each determination. There was some variation in the timing of the withdrawals for technical reasons. On two occasions more than one dose of glucagon was administered. The coma was considered clinically terminated when the patient was able to respond verbally to questions or was able to take fluids by mouth with minimal difficulty.

In addition, seven of the patients had a series of three tests in which glucagon was administered on days when they had not received insulin. In each instance the first of the three tests was performed the day before insulin coma therapy was instituted, the second on the day after they had reached the highest insulin dose in the series of comas and the third on the day following the last insulin coma treatment. A fasting specimen of blood was withdrawn and the glucagon was administered as a rapid intravenous dose. For the tolerance tests the dose was always 0.01 mg. per kg. of body weight.[3] Blood samples were then withdrawn at 10, 20, 30, 45, 60, and 120 minutes.

Early in the work the determination of the blood sugar

[1] This investigation, under the direction of Dr. John C. Whitehorn, was supported by a research grant (M-1085) from the National Institute of Mental Health of the National Institutes of Health, Public Health Service.

[2] The glucagon employed in this study was kindly supplied by Dr. W. R. Kirtley of Eli Lilly and Company. Portions of lots numbered 208-158B-214, 208-158B-214A, and 258-234B-30A were included.

[3] Throughout the paper the glucagon dosage is reported in terms of total milligrams of solids in the glucagon solution. In the lots of glucagon employed in the study about 50 per cent of the solids was actually glucagon.

was performed by the method of Folin (7). The later determinations were performed by the Nelson modification of the Somogyi method (8). In each case the determination was performed on 1 cc. of whole blood. All of the Somogyi determinations were done in duplicate. Most of the values reported for blood sugar are reported as milligrams change in blood sugar which tends to minimize the differences due to method.

RESULTS

Clinical findings

On the 97 occasions when the glucagon was administered intravenously the dose of glucagon varied from 0.003 to 0.3 mg. per kg. of body weight. However, most of the time the dose was between 0.01 and 0.1 mg. per kg. of body weight. On 95 of the 97 occasions the coma was clinically terminated. On two occasions the patient did not awaken. Both of these occurred in the same patient. The dose of glucagon in these two instances was 0.01 and 0.04 mg. per kg. of body weight. On one of these occasions the patient also failed to awaken with intravenous glucose and had a prolonged coma. Successful terminations were accomplished on this patient on other occasions.

On the 22 occasions when glucagon was administered intramuscularly the dose of glucagon varied from 0.02 to 0.1 mg. per kg. of body weight. Nineteen of these terminations were clinically successful. On 3 occasions the patients failed to awaken and responded to intravenously administered glucose.

Glucagon alone was administered subcutaneously on 11 occasions with 7 awakenings and 4 failures. Glucagon was also administered subcutaneously with the addition of 75 turbidity reducing units of a hyaluronidase preparation 11 times. There were 8 successful awakenings and 3 failures.

The average awakening time for all of the intravenous terminations was 13.5 minutes with a range of 4 to 22 minutes. The average awakening time for all of the intramuscular terminations was 16.9 minutes with a range of 7 to 24 minutes. In computing these averages an awakening time of 30 minutes was considered a failure. It was felt that there were insufficient successful subcutaneous terminations to allow for a meaningful average.

At no time was any adverse effect noted either locally or generally following the administration of glucagon.

Characteristics of blood sugar response

The blood sugar levels just prior to the administration of glucagon averaged 15.0 mg. per cent by the Folin method (7) and 5.0 by the Nelson modification of the Somogyi method (8).

Invariably there was a rise in the blood sugar by the time the first blood specimen was obtained following the administration of the glucagon. This usually occurred at 5 minutes and demonstrates the rapidity of the onset of the action of glucagon.

When the glucagon was administered intravenously the average time of the maximal response of the blood sugar was 25.2 minutes, with a range of 17 to 40 minutes. When given intramuscularly the average time of the maximal response was 31.6 minutes with a range of 15 to 56 minutes.

The maximal rise in blood sugar averaged 33.6 mg. per cent with a range of 8 to 59 mg. per cent when the glucagon was given intravenously. When given intramuscularly the average rise was 22.2 mg. per cent with a range of 6 to 48 mg. per cent. The actual rises were probably a little higher, in view of the probability that the highest point was missed because of relatively infrequent blood specimens.

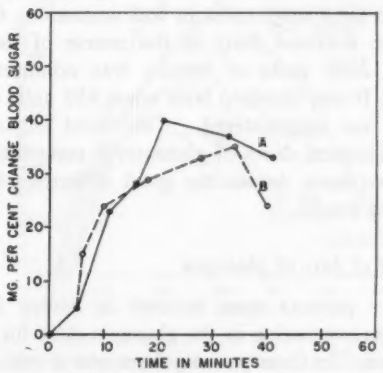

FIG. 1. THE EFFECT OF IDENTICAL DOSES OF GLUCAGON UPON THE BLOOD SUGAR IN THE PRESENCE OF COMAS OF EQUIVALENT DEPTH PRODUCED BY DIFFERENT DOSES OF INSULIN

Both curves were obtained in the same patient with a glucagon dose of 0.05 mg. per kg. of body weight. A—Following the administration of 2,000 units of insulin. B—Following the administration of 850 units of insulin.

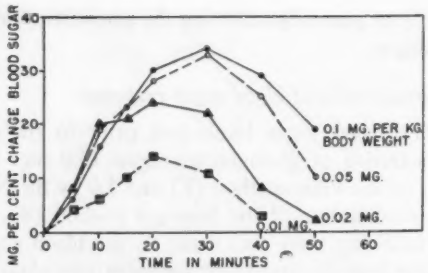

FIG. 2. THE AVERAGE RESPONSES TO A VARIETY OF GLUCAGON DOSES IN FIVE PATIENTS

A total of 35 tests is included, with equivalent doses averaged. In each case the number represents the dose of glucagon in mg. per kg. of body weight.

Effect of dose of insulin

As a patient progresses through a series of insulin comas there is a wide variation in the dose of insulin required to produce a coma of equivalent depth. In terminating insulin comas with glucagon it became clinically evident that the effectiveness of a given dose of glucagon did not depend on the dose of insulin but rather on the degree to which that dose had been effective in producing coma.

Figure 1 records the blood sugar responses in the same patient on two occasions. Each time the dose of glucagon was 0.05 mg. per kg. of body weight administered intravenously. The patient was in third stage coma in both instances. Curve A was obtained early in the course of therapy when 2000 units of insulin was administered. Curve B was obtained later when 850 units of insulin was administered. The blood sugar rise with identical doses of glucagon is comparable in the two cases despite the great difference in the dose of insulin.

Effect of dose of glucagon

Five patients were selected as having had a sufficient variation in the glucagon dose for comparison. In these patients there was a total of 35 curves obtained where the glucagon had been administered intravenously. Whereas very small doses of glucagon would terminate comas successfully, larger doses evoked a greater and more sustained blood sugar response. This was true for each of the 5 patients. Figure 2 is summary of all 35 curves in 5 patients with similar doses averaged. One notes the clear relationship between the dose of glucagon and the amplitude and duration of the response.

Effect of route of administration

There was a consistently greater rise in the blood sugar following the administration of an intravenous dose of glucagon during the course of coma than the rise obtained following an intramuscular dose. The difference in comparable situations was usually evident in the results five minutes after the administration of the drug. The duration of action did not appear to be different as a result of a different route of administration.

Multiple doses of glucagon

Figure 3 records the blood sugar response to 0.05 mg. of glucagon per kg. of body weight administered intravenously at 0, 20, and 42 minutes. In each case the repeated dose evoked a response, as evidenced by the prolongation of the rise in the blood sugar beyond the point that was obtained in any patient when one dose of glucagon was given. Another patient received two doses of glucagon at 30-minute intervals with a similar result.

Effect of a course of insulin coma on response to glucagon

In each of the 7 patients on whom the 3 glucagon tests were performed (prior to insulin, on the day following the highest dose of insulin, and

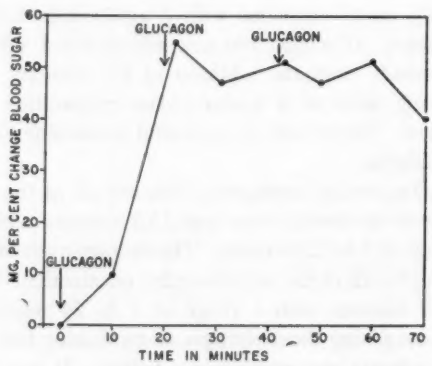

FIG. 3. THE RESPONSE TO MULTIPLE DOSES OF GLUCAGON

The dose of glucagon was 0.05 mg. per kg. of body weight administered at 0, 20, and 42 minutes.

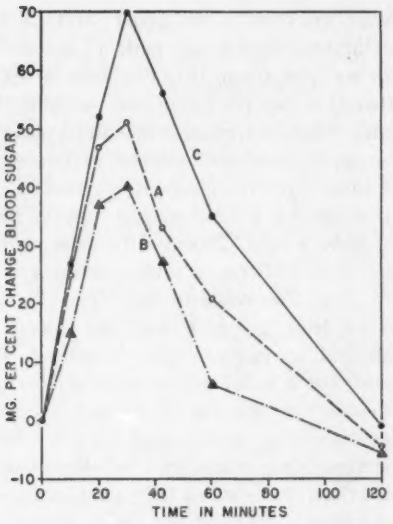

FIG. 4. THE AVERAGE CHANGE IN THE RESPONSE TO GLUCAGON DURING THE COURSE OF INSULIN COMA THERAPY IN SEVEN PATIENTS

The dose of insulin for each curve was 0.01 mg. per kg. of body weight. No insulin was administered on the day of the test. A—The day prior to the institution of insulin therapy. B—The day following the maximal dose of insulin. C—The day following the last insulin treatment.

on the day following the last insulin treatment), it was noted that the maximal rise was greatest in the curve obtained at the termination of the course of insulin treatments. These results are illustrated in Figure 4 which represents the average of these curves for the seven patients. For the differences in the level attained before insulin and following the completion of therapy $P = < 0.02$ at the 30 and 45-minute intervals. The findings are taken to be suggestive of increased response to glucagon developing during the course of insulin coma therapy.

A comparison between the blood sugar response to glucagon in a fasting state and in insulin coma

There were 5 patients who had glucagon administered prior to the onset of insulin coma therapy with suitable blood sampling and who had a large enough number of comas terminated with glucagon with sufficient variation in glucagon dosage to form an adequate basis for comparison. A total of 35 intravenous terminations of coma accompanied by blood sampling was performed on this group. All of the tests before insulin therapy were performed with 0.01 mg. of glucagon per kg. of body weight.

None of the patients achieved a comparable rise in blood sugar following the administration of glucagon in insulin coma when the dose of glucagon was the same as was employed for the tests before insulin. One patient achieved a greater rise in blood sugar following termination on 7 occasions when the dose of glucagon was between 0.05 and 0.1 mg. per kg. of body weight. However, this patient had the unusually low maximal rise of 26 mg. per cent on his test prior to insulin coma therapy. The other 4 patients failed to produce as great a rise in the blood sugar in the termination of insulin coma on 26 occasions as they did on the previous test. The glucagon dosage varied from 0.003 to 0.1 mg. per kg. of body weight, each patient having received the dose of 0.1 mg. per kg. of body weight on at least 1 occasion.

One of these 4 patients received a dose of 0.2 mg. per kg. of body weight on 1 occasion. The rise in blood sugar following the termination of insulin coma with this dose was greater than that obtained on the test performed prior to insulin

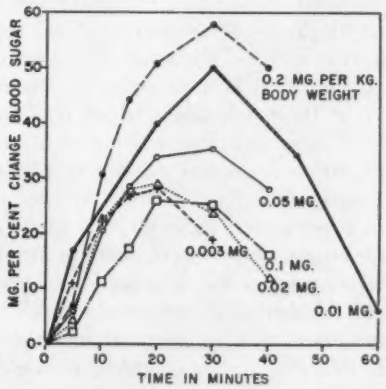

FIG. 5. A COMPARISON OF THE RESPONSE TO VARYING DOSES OF GLUCAGON WHILE IN INSULIN COMA WITH THAT OBTAINED PRIOR TO THE INSTITUTION OF INSULIN THERAPY

All curves represent the responses of one patient. The heavy line is the result of the tolerance test performed prior to the institution of insulin therapy. The other curves were obtained from studies performed while the patient was in insulin coma. The numbers represent the dose of glucagon in mg. per kg. of body weight.

therapy. Figure 5 demonstrates the rise achieved with each dose of glucagon in the termination of insulin coma in this patient as well as the results of the test performed prior to insulin coma therapy.

DISCUSSION

The fact that glucagon is capable of causing a rise in the blood sugar following the administration of moderate doses of insulin is well documented. The demonstration that glucagon is capable of raising the blood sugar following the massive insulin doses employed in this study is a tribute to its extraordinary physiological potency. In a sense this is not surprising for there is no known direct antagonism between insulin and glucagon. The localization of the main if not the sole action of glucagon at the site of liver phosphorylase (9) where insulin has no direct effect would seem to indicate that glucagon would continue to pour glucose into the blood stream independent of the action of insulin.

However, once the glucose is liberated into the blood stream the glucagon no longer has any influence over it and the actions of insulin would tend to remove it. Since the glucose is being removed from the blood stream at a rapid rate, the level of blood glucose is not a good index of the amount of glucose being liberated from the liver through the action of glucagon. The level of glucose in the blood is a reflection of the relative efficacy of those substances which tend to raise the blood sugar and those which tend to lower it. The interrelation of other substances, particularly those elaborated by the pituitary and adrenal glands, is not clear. However, one would expect that the effects of these hormones are manifesting themselves prior to the introduction of glucagon.

From the foregoing, one would conclude that the amount of glucose liberated into the bloodstream following a dose of glucagon would be at least as high during insulin hypoglycemia as when administered without insulin, even though this would not be reflected in the blood sugar levels. Theoretically this concept would appear to be sound. Some of our data would make one question this simple explanation of the relative actions of glucagon and insulin. There is agreement that a relatively small dose of glucagon will cause a maximal rise in blood sugar and further increases in dosage will have no additional effect (3, 4, 10). In preliminary studies on patients not receiving insulin we have found that this dose is definitely less than 0.01 mg. per kg. of body weight. In the presence of insulin hypoglycemia an increased dose of glucagon caused an increased response in the blood sugar over the entire range studied. This was true up to a dose of glucagon of 0.2 mg. per kg. of body weight, 20 times the dose mentioned above. It is difficult to understand why insulin should alter this relationship. It is likely that increased liver glycogen and low blood sugar would tend to increase the amount of glucose liberated, but it is difficult to see how these factors could change the amount of glucagon necessary to produce maximal activation of the phosphorylase mechanism. One would tend to believe that there are additional facts which have a rather important bearing on this situation, but which are not as yet available.

Further evidence that the dose of glucagon necessary to produce a maximal response increases as a result of the effect of insulin is provided by the result of the administration of multiple doses of glucagon. Staub and Behrens (4) have shown in cats that a second dose of glucagon, administered after 25 minutes, had an effect if the first dose had not produced maximal stimulation. If the first dose was sufficient to produce maximal stimulation there was no response to the second dose. We have noted an appreciable response to the second dose even though the first dose was 0.05 mg. per kg. of body weight. Without insulin this dose would have been more than sufficient to produce a maximal response.

The response of the blood sugar to a given dose of glucagon was definitely less during the course of insulin hypoglycemia than prior to the administration of insulin. This is most likely due not to a decrease of the amount of glucose liberated from the liver but rather to its rapid removal from the blood stream. In spite of this our data leave open the theoretical possibility that if sufficient glucagon were given the response would have been even greater during insulin hypoglycemia. This actually occurred in the 1 patient who had a dose of 0.2 mg. of glucagon per kg. of body weight.

It is clear that the response to an intravenous dose of glucagon is greater than that to an intramuscular dose. This is manifested mainly by the

difference in the maximal rise but also by the rapidity of the rise. On clinical grounds the subcutaneous route seems to be considerably poorer than either of the other routes.

It is difficult to evaluate fully the difference in response between patients because of the number of variables involved and particularly because of the problems involved in adopting standards for estimation of the coma. However, the range of variation does not appear to be striking in comparison with the range of response with other carbohydrate tolerance tests and with the range of response to glucagon in patients not receiving insulin.

The change in sensitivity to glucagon during the course of insulin comas also is difficult to evaluate. The weight of these patients tended to increase as a result of the insulin therapy, and the dose of glucagon was administered on the basis of body weight. However, it is not believed that the increase in the dose of glucagon explains the difference, as all of the doses probably produced a maximal response. Neither is the difference due to the fact that the last test was performed on the day after the patient received insulin therapy, since there was a difference between the results of tests performed at the time of the highest insulin dose and those performed at the end of therapy. The highest dose of insulin is almost invariably given very early in therapy. Root (11) has reported an increase in sensitivity to glucagon in rats upon chronic administration. During the course of insulin coma one would expect that the body is producing more glucagon than normally. Perhaps one might view this as a chronic administration of large doses of glucagon and conclude that the increased sensitivity is analogous to the increased sensitivity obtained by Root. The exogenous glucagon administered in the termination of insulin comas may also have played a role, although this phenomenon also occurred in patients who had very few glucagon terminations. The increased sensitivity may well be due to increased liver glycogen stores produced by the insulin.

One is struck with the apparent innocuousness of the glucagon. At no time did any noxious reaction occur which could be attributed to the glucagon.

It would appear that glucagon is a satisfactory drug for the routine termination of insulin comas when given intravenously. Further study is indicated to see whether the intramuscular route will be satisfactory. The addition of a hyaluronidase preparation may well make this feasible. There are several advantages to the use of glucagon for this purpose. One is the decreased volume of injection and decreased viscosity as compared with glucose for intravenous administration. Another advantage is the absence of local reactions, such as thrombosis, at the site of injection. When compared with termination by tube feeding it is less time consuming, technically easier, faster, appears to have a decreased incidence of gastro-intestinal disturbances and decreases the danger of aspiration of gastric contents. One other possible advantage which has yet to be demonstrated is that of decreased weight gain during the course of insulin coma therapy, for excessive weight increase occurs in some patients. Since we are employing the patient's own glucose to terminate the coma this may prove to be true. A much larger series will have to be accumulated to substantiate this possibility.

One disadvantage in the use of glucagon in the routine termination of insulin coma is the necessity that the patient take food by mouth shortly after awakening to prevent his lapsing back into coma. This is also true, however, when intravenous glucose is used for termination. The awakening is not as rapid after glucagon as it is after intravenous glucose where the patient awakens within a few minutes, a fact which may possibly be advantageous (12).

SUMMARY

1. Glucagon has been administered on 141 occasions to patients in insulin coma.

2. Within the dosage limits explored the greater the dose of glucagon the greater was the resultant rise in blood sugar. This was true with doses of glucagon that markedly exceeded the dose that would ordinarily be expected to produce a maximal response.

3. The intravenous route was found to be superior to the intramuscular route of administration. The subcutaneous route was much poorer than either of the other routes.

4. Responsiveness to glucagon increased during the course of insulin coma therapy.

5. Glucagon was usually successful in terminating insulin coma and it is suggested that glucagon has some advantages as a means of routine termination of insulin coma and that a further clinical trial is warranted.

ACKNOWLEDGMENTS

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WEIGHT GAIN FROM SIMPLE OVEREATING. II. SERUM LIPIDS AND BLOOD VOLUME[1]

By JOSEPH T. ANDERSON, ADRIAN LAWLER, AND ANCEL KEYS

(From the Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis, and Mental Health Research Laboratory, Hastings State Hospital, Hastings, Minn.)

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Many questions about the processes and events in the reduction of obesity in man are still obscure or even unexplored, in spite of a very large number of clinical and experimental studies. The reverse situation, the production of obesity in man, has had practically no detailed attention except for scattered clinical observations in rare pathological states. That obesity develops whenever a positive calorie balance is maintained over a long period of time, is axiomatic but not very informative.

Positive calorie balance may, of course, reflect increased calorie intake, decreased output, or both, and the details of metabolism and physiological adjustments are not necessarily the same in these several conditions. It would be expected, in fact, that the effects of a sharp reduction in physical activity without change in the diet must be quite different from those of increased diet and no alteration in activity. In the "natural" (spontaneous) development of obesity in organically healthy man which is so common in our modern society, alterations both in calorie intake and output are probably involved and, in addition, the character of the diet is often changed. What factors, then, are responsible for the various morphological, biochemical, and physiological differences between the person before and after the development of obesity?

Animal experiments on these questions are useful, of course, but the relationships in the production of obesity in the rat or other laboratory animals are not quantitatively transferable to the human situation. We have elected to begin the study of these questions in man by the experimental production of obesity by simple overfeeding without substantial change in physical activity, using for this purpose a diet in which the calorie increase is provided one-third by fat and two-thirds by carbohydrate. This means, in effect, the kind of dietary change that results when there is no change in the diet except for more liberal use of bread and potatoes, together with such fat as is ordinarily eaten with these. The proportion of fat in the diet is thereby not increased and actually is somewhat reduced. This is important because we know that increasing the proportion of fat in the diet will of itself elevate the blood cholesterol concentration, and one of our main concerns was to separate the effects of fattening and the total calorie intake from the effects of increasing the participation of dietary fat in the total metabolism. Following an initial standardization period of six weeks, overfeeding of 20 men under controlled conditions for 20 weeks resulted in an average body weight gain of 10.6 kg. (23 lbs.), and gains of 19.7 to 22.2 kg. (43 to 49 lbs.) in the four men who were most successfully persuaded to eat more than their previous wont. The character of the tissue gained has been reported by Keys, Anderson, and Brozek (1). The present paper reports the findings on the serum lipid concentrations, the blood volume, and the total circulating lipids.

METHODS

Subjects. The subjects were 20 schizophrenic men patients, aged 33 to 45, of the Hastings State Hospital who appeared to be free from metabolic peculiarities (1). Some of their relevant characteristics are summarized in Table I. They were of average to moderately tall stature (5 feet 5 inches to 6 feet 3 inches, average 5 feet 10 inches) and were not remarkable in their initial condition relative to fatness though they tended to be a little thin. In terms of relative body weight, according to the age and height standards customarily used in the United States, Medico Actuarial, 1912 (2), they averaged 90 per cent (range 73 per cent to 113 per cent).

The diet. During the preliminary standardization period (of six weeks), the food consumption of each man was measured and adjusted in an effort to keep weight constant and to discover the individual balance points. The diet was primarily the regular hospital diet, with some alterations to make for simplicity of manipulation

[1] Aided by research grants from the National Dairy Council, Chicago, The Nutrition Foundation, New York, and the Williams-Waterman Fund, New York.

and quantitative measurement. The diets which most closely approximated calorie equilibrium in the 20 individuals averaged 2800 calories and contained 125 gm. of protein, 305 gm. of carbohydrate and 120 gm. of total fats, 39 per cent of the calories coming from the latter source. The diet was fully adequate in vitamins and minerals.

For the period of experimental overfeeding this basic diet was supplemented by larger offerings with encouragement to eat more at the regular meals, and by providing between-meal snacks. The supplementary food consumption averaged 108 gm. of carbohydrate and 22 gm. of fat. The mean protein consumption was the same as in the pre-fattening period although individual subjects made changes in both directions. Food was given in measured portions and all rejections were weighed. Samples of the pooled basic diet and of the supplements were analyzed.

Methods. Blood studies were made on two occasions in the last 3 weeks of the preliminary standardization period and at five-week intervals during overfeeding. The final tests were made at 21 and 23 weeks. All bloods were drawn at rest in the post-prandial state. Hematocrit measurement and serum separation were carried out at once and the other analyses started within a few hours, the serum remaining under refrigeration meanwhile. All analyses were done in duplicate.

Serum cholesterol was measured with the Liebermann-Burchard reaction applied to the Bloor extract (3). Four fresh cholesterol standards were included in each batch of samples and readings were made on the Evelyn photoelectric colorimeter (660 millimicron filter) at the time of maximum color development at 25° C. This method has more recently been replaced by a slightly modified form (4) of the method of Abell, Levy, Brodie, and Kendall (5) which involves saponification prior to the eventual estimation with the Liebermann-Burchard reagent. The latter values can be predicted from the former by multiplication by the factor 0.836 (6).

Plasma volume was determined by dilution of Evans Blue dye (T-1824) using essentially the method of Gibson and Evelyn (7). The solution injected contained approximately 15 mg. of Evans Blue, 500 mg. NaSCN and 1 gram antipyrine in 20 ml. isotonic saline. Plasma volume was computed from heparinized plasma samples taken at 20, 30, and 40 min. Hematocrit determinations were done by centrifuging the heparinized blood in Wintrobe tubes for 1 hour at 800 times gravity.

The S_f 12–20 lipoproteins of serum are those components which when suspended in a medium of 1.063 specific gravity and subjected to the intense gravitational field of the ultracentrifuge undergo flotation at rates between 12 and 20 Svedberg units. The determination of these fractions was done in the Spinco model E ultracentrifuge using techniques described by Gofman and his associates (8–10), as modified by the Committee on

TABLE I

Pre-fattening characteristics of the subjects and body weights during overeating

Subject	Age* (*years*) Start	Relative body weight‡ (%) Start	Body weight (*kg.*) Start	5 wks.	10 wks.	15 wks.	20 wks.
Er	34	101	71.9	75.8	84.4	87.7	94.2
Lo	38	99	68.2	71.4	78.5	82.2	89.2
Pro	45	113	90.2	93.4	98.5	104.5	111.1
He	35	89	67.8	72.3	77.4	81.2	87.5
Sa	39	86	75.7	80.8	85.5	88.2	93.5
Be	35	91	67.0	73.3	78.1	79.6	82.2
Br	41	86	66.6	70.8	74.1	76.3	80.8
Fo	39	101	69.3	74.0	77.6	79.5	82.4
Pu	34	90	76.2	79.0	83.9	86.4	86.8
Do	42	90	60.5	62.8	64.9	66.6	69.9
An	41	75	56.6	57.8	60.4	61.6	64.4
Hu	40	73	51.5	52.5	54.5	55.1	57.2
Su	33	109	73.6	74.5	74.9	76.7	79.2
Hi	45	85	68.4	68.2	71.1	71.9	73.8
To	44	98	88.5	91.7	94.7	95.3	93.2
Pra	41	74	56.1	57.5	61.9	63.4	60.7
We	33	86	63.9	67.1	67.3	67.9	67.8
Ma	43	80	65.6	67.6	69.5	69.4	69.5
Dr	44	77	56.3	59.5	59.9	60.0	59.0
Mu	33	99	73.3		75.2	76.4	75.8
No.	20	20	20	19	20	20	20
Mean	39.0	90.1	68.4	71.0	74.6	76.5	78.9
S.D.†	±4.3	±11.3	±9.9	±10.7	±11.4	± 12.4	±13.9

* Nearest birthday.

† Standard Deviation $= \sqrt{\frac{N\Sigma X^2 - (\Sigma X)^2}{N(N-1)}}$.

‡ As percentage of the average for equal height and age in the standard Medico-Actuarial tables.

TABLE II

Serum lipids and blood characteristics

Subject	Serum cholesterol (mg. per 100 ml.)					S_f 12–20 (mg. per 100 ml.)		Plasma volume (liters)			Hematocrit (%)		
	Start	5 wks.	10 wks.	15 wks.	20 wks.*	10 wks.	20 wks.	Start*	10 wks.	20 wks.*	Start	10 wks.	20 wks.
Er	205	252	249	225	225	32	66	2.72	3.20	2.58	40.6	40.2	41.4
Lo	273	293	384	370	376	46	90	2.88	3.29	2.93	44.7	48.2	46.5
Pro	273	297	288	330	373			3.95	3.60	4.00	43.8	44.0	42.4
He	281	322	310	317	310		38	2.71	2.51	2.55	47.0	50.6	49.1
Sa	248	259	256	258	244	21	72	3.17	2.90	3.53	43.7	49.7	45.2
Be	191	220	234	226	188	24	38	2.72	3.34	3.38	47.7	49.8	48.6
Br	220	205	242	206	237	22	27	2.83	2.77	2.82	46.7	50.4	47.9
Fo	239	283	284	270	274	13	23	2.83	3.08	3.07	46.4	45.4	46.4
Pu	192	220	233	215	206	15	37	3.55	3.52	3.75	41.8	46.3	46.6
Do	221	254	226	259	226	3		2.78	2.97	2.49	43.8	46.0	46.5
An	197	207	202	203	216		17	2.62	2.77	2.86	45.4	48.4	47.0
Hu	181	190	208	209	226	29	57	2.54	2.80	2.56	45.6	46.6	45.2
Su	202	218	204	228	230	14	59	2.84	3.00	3.11	44.8	45.2	45.0
Hi	272	292	312	306	322	18	24	3.08	2.83	3.33	41.1	44.0	42.3
To	223	238	219	223	218	13	33	3.62	3.66	3.93	45.9	45.5	46.9
Pra	227	274	250	246	248		18	2.59	2.99	2.54	45.8	48.1	46.2
We	193	186	204	190	193		58	3.04	3.16	2.88	42.6	46.2	44.8
Ma	284	294	332	296	311		41	2.46	2.96	2.92	51.0	53.1	52.4
Dr	221	224	228	222	220		18	2.84	2.76	2.76	44.9	46.4	45.4
Mu	227		186	226	200	31	16	2.97	3.24	3.33	45.7	45.4	45.0
No.	20	19	20	20	20	13	18	20	20	20	20	20	20
Mean	228.5	248.8	252.6	251.2	252.2	21.6	40.7	2.936	3.068	3.067	44.95	46.98	46.04
S.D.†	33.2	40.8	51.1	48.9	56.7	11.03	21.72	0.382	0.307	0.469	2.40	2.89	2.48

* Mean of two determinations.
† Standard deviation.

Lipoproteins and Atherosclerosis, National Advisory Heart Council.

In this laboratory the area measured on the ultracentrifugal pattern for determining the S_f 12–20 concentration is bounded by lines drawn at exactly the positions where peaks for S_f 12 and S_f 20 particles would fall. In some laboratories the area is measured between S_f 11 and S_f 21 lines. Such a method would give higher concentrations of S_f 12–20 fraction than we have reported. Duplicate determinations of S_f 12–20 lipoprotein fraction in each of 10 serum samples gave a coefficient of reliability of 0.75 with a standard error of measurement of 6.8 mg. per 100 ml. Twenty men were tested twice within two weeks for S_f 12–20 lipoprotein and a coefficient of reliability of 0.62 was found with a standard error of measurement of 7.7 mg. per 100 ml.

RESULTS

The attempt to persuade the subjects to overeat met with great success in some cases and with little response in others. The elicited increases in calorie intake ranged from 39 per cent to 8 per cent. The mean calorie intakes (± standard deviation) in the last 4 weeks of the preliminary period and in the overfeeding period were 2800 (± 340) and 3430 (± 490) calories, respectively. The effects of 20 weeks of this dietary regimen on serum cholesterol, S_f 12–20 lipoprotein, plasma volume and hematocrit are presented in Table II.

The changes in body weight caused by overnutrition are given in Table I. The greatest gain in body weight was 22.2 kg. in 20 weeks and 8 men gained more than 13 kg. each. In Table III the mean responses of these 8 men to the overnutrition regimen are compared with the mean responses of the group of 9 men who gained least. For most of the men the calorie intake was practically constant from week to week in the overnutrition period and the rate of weight gain was quite uniform throughout the 20 weeks.

The men who gained most weight increased on the average about 40 mg. per 100 ml. in serum cholesterol concentration during the first 10 weeks of overnutrition (25 mg. in the first 5 weeks). During the last 10 weeks these men continued to overeat and to gain weight at nearly the same rate but no further change in cholesterol concentration occurred. The men who gained least weight had only a small average increase in serum cholesterol concentration which is not statistically significant. The calculated quantities of total circulating se-

TABLE III

The responses of two groups of men to attempted overfeeding for 20 weeks *

	High gain group (8 men)	Low gain group (9 men)
Body weight (*kg.*)		
At start	72.1	66.3
Gain in first half	9.9 ± 0.6	3.5 ± 0.4
Gain in last half	8.1 ± 1.0	0.8 ± 0.5
Serum cholesterol concentration (*mg./100 ml.*)		
At start	241	226
Gain in first half	40 ± 11	13 ± 9
Gain in last half	−3 ± 13	3 ± 5
Total circulating serum cholesterol (*grams*)		
At start	7.23	6.50
Gain in first half	1.45 ± 0.64	0.71 ± 0.35
Gain in last half	0.04 ± 0.75	0.12 ± 0.34
Plasma volume (*ml.*)		
At start	2976	2885
Gain in first half	111 ± 133	159 ± 79
Gain in last half	22 ± 139	−3 ± 98
Blood volume (*ml.*)		
At start	5405	5267
Gain in first half	454 ± 241	456 ± 161
Gain in last half	−90 ± 199	41 ± 201

* The "high gain group" includes the 8 men who gained between 13 and 22 kg., the "low gain group" includes the 9 men who gained between 2 and 6 kg. Mean values and standard errors (±).

rum cholesterol (mean ± standard deviation) for all 20 men were 6.72 ± 1.42, 7.74 ± 1.74, and 7.76 ± 2.34 gm. at the start, 10 weeks, and 20 weeks, respectively.

The concentration of the S_f 12–20 lipoproteins in the serum increased from the tenth to the twentieth week of overeating in all but one of the 12 men for whom these measurements are available on both occasions. The mean change was a rise of 22.0 mg. per 100 ml., and this is significant with a probability for chance occurrence of 0.002, the t value being 3.97. During this period the serum total cholesterol values for these same 12 men fell by an average of 5.4 mg. per 100 ml., which is statistically insignificant. There was no correlation between the changes in S_f 12–20 and those in cholesterol in this interval, the coefficient of correlation being $r = 0.047$. We have no explanation for this finding.

The gain in body weight was accompanied by some tendency to increase in the plasma and whole blood volume. The individual values of plasma volume and hematocrit are listed in Table II. The mean plasma volume increased by 0.13 liters in the first 10 weeks ($P = 0.05$) and remained constant thereafter. The mean hematocrit increased in the first 10 weeks and subsequently decreased by a smaller amount so that it ended above the starting

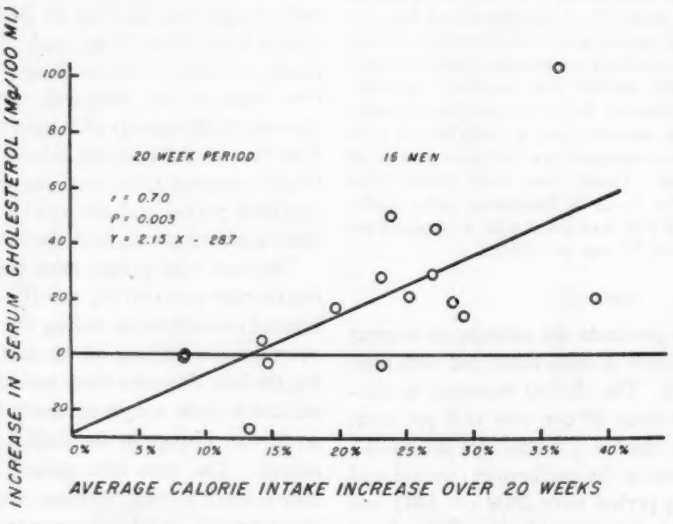

FIG. 1. RELATION OF THE INCREASE IN SERUM CHOLESTEROL CONCENTRATION TO THE AVERAGE INCREASE IN CALORIC INTAKE

TABLE IV

Regression of serum cholesterol elevation above the initial level on rate of body weight gain (equation passing through the origin) in men while overeating

	5 Weeks	10 Weeks	15 Weeks	20 Weeks
1. Number of subjects	19	19	20	20
2. Weight gain rate in 5 preceding weeks				
Mean (*gm./day*)	85	101	54	69
Standard deviation (*gm./day*)	±47	±63	±40	±85
3. Serum cholesterol elevation above initial level				
Mean	8.8%	11.7%	9.6%	9.8%
Standard deviation	±8.0%	±10.4%	±9.7%	±12.6%
4. Regression coefficient of item 3 on item 2, equation passing through the origin	0.085	0.111	0.157	0.104
Standard error	±0.021	±0.017	±0.028	±0.024

value. All of these differences were statistically significant. Blood volume computed from plasma volume and hematocrit increased significantly in the first half of the experiment and thereafter decreased by a small amount which was not statistically significant. The mean values of blood volume (± standard deviations) for the 20 men were 5.32 ± 0.63, 5.79 ± 0.54, and 5.68 ± 0.87 at the start, middle and end, respectively. Plasma volume, blood volume and hematocrit mean values were all significantly higher at all times during the weight gaining process than at the start.

The increase in plasma and blood volumes occurred equally in the high-gain and low-gain groups (Table III). These changes in the subgroups do not reach statistical significance chiefly because the number of men in each group is small. The net conclusion of the experiment with respect to plasma and erythrocytes is an indication that overeating caused an increase in both but no evidence that the largest blood increases were associated with the largest body weight gains.

Since all the men were urged to eat as much as possible and each man chose his own rate of eating we do not have two well-defined groups of "gainers" and "non-gainers" but rather a continuous series extending from the man who gained least, 2.5 kg., to the man who gained most, 22.2 kg. Data of this type are not well suited to analysis by grouping and are better treated by correlation methods.

The relationship between increase in serum cholesterol concentration in 20 weeks and increase in rate of eating is presented in Figure 1. There is a marked tendency for the men who increased their calorie intake most to experience the greatest rise in serum cholesterol. The slope of the regression line indicates that a 1 per cent increase in calorie intake continued for 20 weeks resulted in an average increase of 2 mg. per 100 ml. in serum cholesterol concentration (standard error 0.6 mg.). The correlation coefficient between these variables is 0.70 and the probability that this correlation could have arisen due to chance alone is 0.003.

In many practical situations information is available concerning body weight changes but nothing is known about food consumption. The question may be raised whether an observed gain in body weight can be used to predict an increase in serum cholesterol. Table IV shows the relationship between body weight gain, expressed as rate of gain during the preceding 5 weeks, and serum cholesterol concentration expressed as percentage increase above the initial value. The restriction is imposed that the regression lines should pass through the origin because constant body weight must theoretically correspond to constant serum cholesterol concentration under the conditions of this experiment. On each occasion of cholesterol determination during overfeeding there was a significant positive regression between the hypercholesterolemia and the rate of body weight gain. Each regression coefficient is the percentage elevation of serum cholesterol above the steady-state level corresponding to 1 gram body weight gain per day. The value was significantly greater than zero at each observation during the overeating period. It is inferred that a rise in serum choles-

terol appeared promptly with overeating, the increase being roughly proportional to the rate of weight gain. But as overeating and gaining weight continued, the serum cholesterol concentration did not tend to change further.

Since determinations of serum S_f 12–20 lipoproteins were made only at the middle and end of the overnutrition period, the change in S_f 12–20 lipoproteins in the last ten weeks was tested for correlation with increase in calorie intake and with gain in body weight. No significant correlation was found in either case. During this latter half of the overnutrition period serum cholesterol increase between periods was likewise unrelated to the degree of overeating or weight gain.

The correlation analysis described attempted to answer the question—what change do overeating and the maintenance of a positive calorie balance produce in serum cholesterol and S_f 12–20 lipoprotein concentrations? It is also pertinent to ask —is there a tendency for men who are overweight to have unusually high concentrations of serum cholesterol or S_f 12–20 lipoproteins?

Among middle-aged men examined by the Laboratory of Physiological Hygiene, 162 maintained constant relative body weight within 5 per cent for 4 years. In this group of men who varied widely in relative obesity but all of whom were close to calorie equilibrium, there was no correlation between serum cholesterol concentration and relative body weight (correlation coefficient = 0.02, total number of tests = 648). The overweight men had no higher serum cholesterol concentration than the underweight men. In the men who were overeating and in positive calorie balance, the relationship was different. Using 79 tests on 20 men during the period of overfeeding, the coefficient of correlation was 0.35 between serum cholesterol concentration and relative body weight. This value is small but it is statistically significant ($P = 0.004$). Among these men the most overweight individuals tended to have the highest serum cholesterol concentrations.

Serum S_f 12–20 lipoprotein was similar to serum cholesterol in relation to relative weight. In the men who were overeating, 31 tests on 19 men gave a coefficient of correlation of 0.40 between S_f 12–20 lipoprotein and relative body weight. Although the coefficient is higher, the probability judgment is less decisive in this case ($P = 0.025$) because the number of tests was smaller. Like serum cholesterol, the S_f 12–20 fraction showed no correlation with relative weight in the men who were stable in body weight, 73 tests on 72 men resulted in a correlation coefficient of 0.08.

After the overfeeding experiment was finished the men were allowed to eat as they pleased without any guidance. Eighteen months later 19 of the 20 men were again observed. Seven men had lost more than 10 kg. since the end of overfeeding and 6 of these were among the 9 men who had gained over 10 kg. in the experiment. They showed an average fall of 20 mg. of cholesterol and 40 mg. of S_f 12–20 lipoprotein per 100 ml. of serum (ranges -57 to -8 and -82 to -10, respectively) from their values at the end of the overfeeding period. Eleven of the former subjects showed smaller weight changes since the overfeeding period, and this group included 7 of the 9 men who had gained least in the experiment. Their serum cholesterol and S_f 12–20 concentrations showed a statistically insignificant tendency to rise slightly (averages of $+15$ and $+10$ mg. per cent, respectively) from their values at the end of the experiment.

DISCUSSION

What caused the observed significant increases in serum cholesterol? It has been repeatedly observed that, in calorie equilibrium, a major change in the proportion of calories provided as mixed food fats tends to produce corresponding changes in the concentration of cholesterol and of S_f 12–20 lipoprotein in the serum (9, 11–13).

In the present experiment the proportion of calories provided by fats was slightly *decreased* during the overfeeding (37 per cent versus 39 per cent), so the present observations might seem discordant with these other findings. However, it will be observed that the absolute amount of fat consumed was increased by an average of 22 gm. per day and such a change in fat consumption under conditions of calorie equilibrium may be expected to produce a rise in serum cholesterol concentration (14, 15).

Consideration of these facts might suggest that the serum cholesterol level tends to reflect the total fat ingestion, probably because cholesterol is a necessary part of the lipoprotein fat-transport system in the blood. In calorie equilibrium this load of fat metabolism is directly related to the

proportion of the total calories consumed as fats but in positive calorie balance the absolute fat intake may be a better measure of the fat metabolism load. But this explanation, without further elaboration, fails to account for the fact that men who are physically active tend, in comparison with less active men in the same population, to have an elevated absolute fat intake but not elevated blood cholesterol values (16). Moreover, when young men greatly increase their energy expenditure by exercise and maintain calorie equilibrium by increasing the diet, the serum cholesterol does not rise in spite of a considerable increase in fat consumption (17).

However, allowance must be made for the fact that when the total rate of energy metabolism is increased, there is normally a parallel increase in the circulatory rate so that the *rate* of fat transport will be increased even though there is no rise in *concentration* of the fat, or lipoprotein, in the blood. Accordingly, it would seem that all of the present data are in harmony with the concept that, other things being equal, the serum cholesterol concentration is determined by the fat transport load per unit of circulation imposed on the blood. At calorie equilibrium this is determined by the proportion of the calories presented as fats and this relationship is not altered by increasing the energy level of intake if calorie equilibrium is maintained by increased exercise which normally also involves a proportionate increase in circulatory rate. In positive calorie balance, the fat transport load is obviously increased, even if the character of the diet is constant. Indeed, if fat storage is taking place, that portion of the fat synthesized from carbohydrate in the liver also adds to the transport load so we might expect some serum cholesterol rise even on a reduced fat diet during the active phase of gaining weight. But, if the calorie excess and the weight gain steadily continue, there is no further increase in the fat transport load and the serum cholesterol should remain constant at its newly raised level. Finally, if calorie equilibrium is now achieved and obesity is steady, the serum cholesterol should be expected to approximate that characteristically associated with the proportion of fat in the diet at calorie equilibrium regardless of whether this equilibrium means steady obesity, steady "normality," or steady leanness.

The fat-transport of relevance would seem to be that from intestine to liver and from liver to fat depots. These avenues are active in overnutrition and in high fat ingestion and are inactive in undernutrition and on a low-fat or fat-free regiment. The transport of fat from the depots to the liver and of fat metabolites from the liver to the muscles for burning is less obviously related to serum cholesterol. In undernutrition the serum cholesterol level usually falls though much fat is being transported away from the adipose depots.

The increase of S_f 12–20 lipoprotein which occurred in the last half of the overfeeding period involved the transfer of only about one-fortieth of the cholesterol of the plasma from one lipoprotein class to another. There is no apparent reason for the difference but this lipoprotein class seems to have exhibited a slower or longer continued response to the increase in food intake than the preponderant cholesterol-bearing fractions.

SUMMARY

Twenty physically healthy schizophrenic men increased their calorie intakes without changing their physical activity. The diet was substantially constant and fully adequate in proteins and vitamins at all times, the extra calories being provided by adding carbohydrates and a small amount (about one-third of the extra calories) of mixed fats to the standard diet. Thus the total fat intake increased though the proportion of calories from fats fell slightly. Average calorie increases for 20 weeks ranged from 8 per cent to 39 per cent and these produced weight gains from 2.5 to 22.2 kg., the average gain being about 0.5 kg. per week.

The average total serum cholesterol concentration rose 20 mg. per 100 ml. during the first 5 weeks of overeating and then remained substantially constant at the same elevated level during the next 15 weeks though weight gain continued at the same rate as during the first five weeks. The rise in serum cholesterol concentration in the various individuals tended to be proportional to the rate of weight gain.

The concentration of the S_f 12–20 lipoprotein fraction in the serum, measured in the ultracentrifuge, tended to increase from the tenth to the twentieth week of overeating though the total serum cholesterol remained constant.

Weight gain tended to be associated with increase in the circulating plasma and blood volume during the first weeks of overeating with no further change thereafter.

A hypothesis as to the role of cholesterol in fat transport is presented which seems to explain: 1) The increase in serum cholesterol on a high fat diet and in overnutrition with a positive calorie balance; 2) The stability of the serum cholesterol during calorie equilibrium or in a steady state of continuing overnutrition; 3) The failure of serum cholesterol to rise when diet calories and energy expenditure are both increased in parallel; and 4) The decrease in serum cholesterol on a low fat diet and in negative calorie balance.

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URINARY HEMOGLOBIN EXCRETION AND RENAL CIRCULATORY DYNAMICS: A STUDY OF THE EFFECT OF L-NOREPINEPHRINE IN THE DOG [1, 2]

By WILLOUGHBY LATHEM [3] AND ALVIN D. BENJAMIN [4]

(From the Department of Internal Medicine, Yale University School of Medicine, and the Veterans Administration Hospital, West Haven, Conn.)

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The passage of protein molecules across capillary walls is governed by the characteristics of the molecule and the membrane and appears to occur by a process of both filtration and diffusion. From a study of these processes within the isolated, perfused hind limb of the cat (2, 3) and from mathematical considerations and other studies (2, 4) it has been suggested that the transcapillary transport of protein and the degree of molecular sieving of protein molecules across membranes during ultrafiltration may be influenced by concentration gradients of protein between filtrate and filtrand and by the rate of filtration of water through the capillary wall. Moreover, it has been suggested that these processes may be influenced by factors which modify the level of hydrostatic pressure within the capillary (2, 5, 6).

Within the kidney these processes have not been subjected to extensive study. That intraglomerular hydrostatic pressure may modify the rate of filtration of protein molecules has been suggested by experiments in which renin, which presumably elevates intraglomerular pressure, has been shown to accelerate the urinary excretion of plasma proteins (7, 8) and hemoglobin (9). A change in pressure has also been invoked to account, at least in part, for the augmentation of protein clearances following the administration of serum albumin intravenously to patients with renal disease (5). Of molecular sieving within the kidney and of the influence of changes in glomerular filtration rate, little is known.

In the present investigation these factors were examined in normal, anesthetized dogs. The rate of urinary excretion and renal clearance of hemoglobin were studied under conditions in which renal hemodynamics and glomerular filtration rate were modified by infusions of the adrenal medullary hormone, l-norepinephrine.

METHOD

A study of the effects of l-norepinephrine was made in 20 female mongrel dogs weighing 7 to 20 kg. All animals were deprived of food, but not water, for 24 hours prior to the test. Anesthesia was induced with sodium pentobarbital, 30 mgm. per kg. intravenously, and was maintained by the administration of 100 mgm. of this drug at intervals throughout the study. An indwelling needle was placed in a femoral artery. Priming and sustaining infusions of creatinine and p-aminohippurate were administered intravenously for measurement of glomerular filtration rate and renal plasma flow, respectively. Along with these substances, solutions of dog hemoglobin were administered intravenously in amounts sufficient to maintain a relatively constant arterial plasma level of hemoglobin of from 212 to 504 mgm. per cent, values well above the renal threshold (10). Priming doses consisted of from one to two grams of hemoglobin in 50 ml. of 5 per cent dextrose and distilled water. The sustaining infusion contained hemoglobin in a concentration of from 430 to 760 mgm. per cent and was made up in 5 per cent dextrose and distilled water. This was administered at a constant rate of from 1 to 2 mgm. of hemoglobin per minute.

After allowing 30 minutes for equilibration of these substances, the urine, collected by urethral catheter, was discarded and the bladder was washed out with distilled water and emptied by manual compression after the introduction of air to facilitate emptying. Three consecutive 10 to 20-minute control clearance periods were then obtained. Following this an infusion of l-norepinephrine (16 μg. per ml.) was administered intravenously at a rate sufficient to elevate mean arterial blood pressure, measured with a mercury manometer, 20 to 50 mm. Hg. This

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[2] The results of this study have been published in abstract form (1).

[3] Present address: Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

[4] This work was submitted to the Faculty of Medicine, Yale University, in partial fulfillment of requirements for the degree of Doctor of Medicine.

TABLE I

Urinary hemoglobin excretion and renal hemodynamics during the infusion of l-norepinephrine *

Dog No.	Wt. Kg.	Procedure	B.P. mm. Hg	HR min.	GFR ml./min.	RPF ml./min.	FF	P_{Hgb} mgm. %	$U_{Hgb}V$ mgm./min.	C_{Hgb} ml./min.	$\frac{C_{Hgb}}{C_{cr}}$ %
3	10	Control	114	120	54	112	.482	250	3.04	1.22	2.28
		Infusion	165	72	41	76	.541	305	4.22	1.38	3.42
7	17	Control	94	140	35	70	.501	249	2.30	0.92	2.65
		Infusion	140	180	21	34	.620	348	4.22	1.12	5.71
8	20	Control	104	120	98	207	.471	212	2.11	0.93	0.99
		Infusion	140	120	85	147	.587	223	4.18	1.87	2.18
9	7	Control	85	160	48	87	.552	504	4.58	0.91	2.09
		Infusion	115	140	20	43	.473	501	3.71	0.74	3.71
10	7	Control	110	160	31	135	.230	394	0.72	0.18	0.61
		Infusion	135	128	39	118	.350	470	2.28	0.48	1.26
11	8	Control	85	130	27	131	.203	354	1.90	0.54	2.04
		Infusion	130	100	8	23	.427	403	1.71	0.43	5.69
12	12	Control	95	140	31	92	.339	317	2.18	0.68	2.20
		Infusion	130	128	26	82	.316	379	4.40	1.16	4.46
13	13	Control	120	132	32	117	.276	308	2.35	0.77	2.44
		Infusion	150	—	17	50	.355	391	6.97	1.79	10.36
14	9	Control	100	132	24	76	.320	336	2.66	0.89	3.83
		Infusion	130	168	13	36	.428	397	3.47	0.91	8.22
15	7	Control	90	124	23	53	.435	395	3.12	0.88	3.90
		Infusion	110	160	15	33	.466	410	4.30	1.06	7.68
16	15	Control	90	156	44	111	.406	244	2.71	1.12	2.56
		Infusion	120	160	38	91	.415	283	3.28	1.16	3.70
19	13	Control	105	132	23	56	.434	244	3.62	1.50	6.54
		Infusion	135	120	12	30	.397	310	3.16	1.03	8.83
31	16	Control	100	112	98	209	.473	460	2.07	0.45	0.47
		Infusion	150	100	88	177	.496	523	3.52	0.68	0.77
32	12	Control	100	92	70	108	.648	301	1.69	0.56	0.80
		Infusion	130	140	62	99	.630	405	3.02	0.75	1.21
17	9	Control	98	148	38	106	.358	285	1.94	0.69	1.81
		Infusion	140	120	27	59	.470	321	2.40	0.76	2.91
		Recovery	70	134	32	75	.422	331	2.79	0.85	2.70
18	9	Control	95	148	37	100	.375	273	2.26	0.83	2.22
		Infusion	130	120	18	43	.499	300	2.76	0.92	7.59
		Recovery	90	140	25	63	.416	301	3.19	1.06	4.08
25	9	Control	95	100	62	94	.655	246	3.96	1.65	2.73
		Infusion	140	132	56	90	.615	323	3.62	1.12	2.04
		Recovery	90	98	57	98	.576	331	5.79	1.74	3.02
33	13	Control	110	120	44	117	.396	477	2.67	0.56	1.28
		Infusion	160	100	32	57	.555	598	5.50	0.92	2.91
		Recovery	75	138	41	90	.459	593	4.25	0.71	1.71
34	10	Control	110	84	69	104	.663	345	1.10	0.32	0.47
		Infusion	160	136	59	90	.655	419	2.40	0.57	0.98
		Recovery	75	138	66	142	.471	406	2.01	0.50	0.76
35	12	Control	120	144	32	82	.394	341	1.25	0.38	1.16
		Infusion	160	128	19	35	.553	450	2.83	0.63	3.55
		Recovery	—	—	19	59	.334	441	1.36	0.30	1.74

* All values are averages of three determinations. L-norepinephrine was administered intravenously during infusion periods. Abbreviations are as follows: BP = mean arterial blood pressure; HR = heart rate, beats per minute; GFR = glomerular filtration rate (creatinine clearance); RPF = renal plasma flow (PAH clearance); FF = filtration fraction (GFR/RPF); P_{Hgb} = plasma hemoglobin concentration; $U_{Hgb}V$ = urinary hemoglobin excretion; C_{Hgb} = clearance of hemoglobin; C_{Hgb}/C_{cr} = hemoglobin-creatinine clearance ratio.

required between 4 and 50 μg. of l-norepinephrine per minute. In most animals the amount necessary to maintain arterial pressure at the desired level increased progressively throughout the test. After elevation of the arterial pressure for approximately 15 minutes the urine was discarded and three consecutive 10 to 20-minute clearance periods were obtained during the pressor response to l-norepinephrine. In each of six animals (17, 18, 25, 33, 34, and 35) three recovery periods were obtained immediately following cessation of the infusion of l-norepinephrine.

Urinary and plasma concentrations of hemoglobin were determined by the method of Evelyn and Malloy (11). Creatinine concentrations in urine and plasma were assayed by the method of Bonsnes and Taussky (12) and PAH levels were determined by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (13). Hemoglobin-saline solutions were prepared according to the method of Amberson, Jacobs, Hisey, and Monke (14). Solutions of hemoglobin prepared in this manner were dialyzed against normal saline at 8°C for 48 hours. After filtration through a Seitz filter the solution was stored in a sterile container at 8°C for periods not exceeding two weeks.

RESULTS

The results of the effects of l-norepinephrine on renal hemodynamics and hemoglobin excretion are presented in Table I and in Figures 1 to 6. An illustrative experiment is presented in Figure 1 (dog 33).

Systemic circulatory response

During the infusion of l-norepinephrine the arterial blood pressure increased from 20 to 50 mm. Hg, with a mean change of 38 mm. Hg. Considerable variation was encountered in the pressor response to l-norepinephrine. Some animals required as much as 50 μg. per minute to maintain the pressure within this range whereas others re-

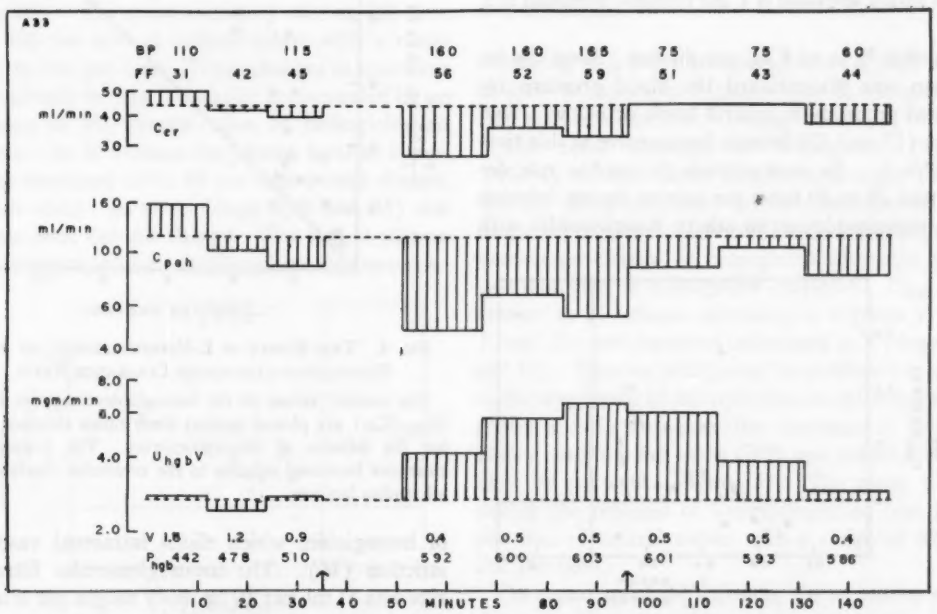

FIG. 1. THE EFFECT OF L-NOREPINEPHRINE ON RENAL FUNCTION AND URINARY HEMOGLOBIN EXCRETION (DOG 33)

Glomerular filtration rate (C_{Cr}-creatinine clearance), renal plasma flow (C_{PAH}), urinary hemoglobin excretion ($U_{Hgb}V$) and mean arterial blood pressure (BP) were determined before, during and following the administration of l-norepinephrine. The drug was given intravenously during the time noted between the arrows. The urine obtained during the first 15 minutes after beginning infusion was discarded because of intrarenal delay. Urinary hemoglobin excretion increased in association with a rise in arterial pressure and plasma hemoglobin concentration (P_{Hgb}, at bottom) and a fall in glomerular filtration rate and renal plasma flow. The filtration fraction increased. Urine flow (V) diminished. After the infusion was discontinued all values returned towards control levels.

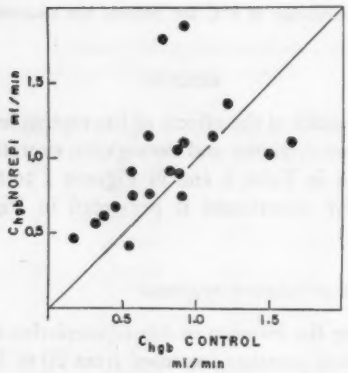

FIG. 2. THE EFFECT OF L-NOREPINEPHRINE ON THE RENAL CLEARANCE OF HEMOGLOBIN

The averaged values for the clearance of hemoglobin during the control periods are plotted against the clearances of this protein during the pressor response to l-norepinephrine. The clearance of hemoglobin increased in 14 studies, decreased in 4, and remained unchanged in 2.

quired as little as 4 μg. per minute. After the infusion was discontinued the blood pressure returned promptly to control levels or below. Two dogs (17 and 33) became hypotensive at this time (Table I). In most animals the cardiac rate decreased 20 to 30 beats per minute during infusion of l-norepinephrine; in others, a tachycardia with

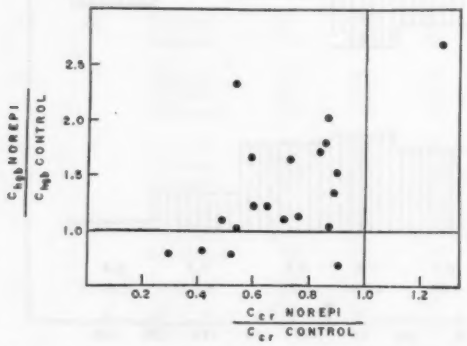

FIG. 3. THE RELATIONSHIP BETWEEN THE CHANGES IN THE CLEARANCE OF HEMOGLOBIN AND THE CLEARANCE OF CREATININE DURING THE PRESSOR RESPONSE TO L-NOREPINEPHRINE

The ratio of the infusion to the control values of the clearance of hemoglobin is plotted against the changes in the creatinine clearance. The clearance of hemoglobin tended to increase during the infusion of l-norepinephrine except in those instances when the reduction in the creatinine clearance exceeded 50 per cent of control values.

varying degrees of irregularity of cardiac rhythm was observed.

Renal hemodynamics

During control observations prior to the infusion of l-norepinephrine the mean renal plasma flow for the entire group of 20 animals was 100 ml. per 10 kg. body weight per minute, a value less than that reported (15) for normal dogs under basal conditions—135 ml. per 10 kg. body weight per minute. The mean renal filtration fraction (GFR/RPF) was 0.43, a value higher than that reported (15) for normal animals — 0.32. These differences may be attributed to the administration

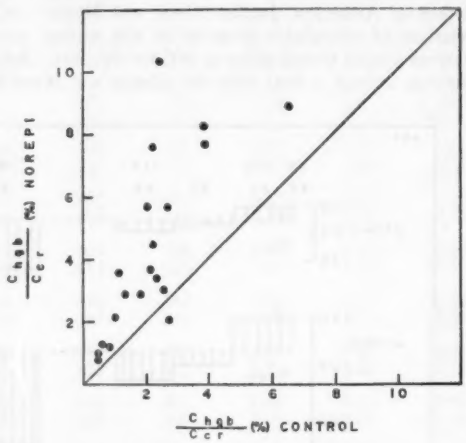

FIG. 4. THE EFFECT OF L-NOREPINEPHRINE ON THE HEMOGLOBIN-CREATININE CLEARANCE RATIO

The control values of the hemoglobin-creatinine ratios (C_{Hgb}/C_{Cr}) are plotted against these ratios obtained during the infusion of l-norepinephrine. The hemoglobin clearance increased relative to the creatinine clearance in all studies but one.

of hemoglobin, which elicits intrarenal vasoconstriction (16). The mean glomerular filtration rate was 41 ml. per 10 kg. body weight per minute, a figure not significantly different from normal values (15).

During infusion of l-norepinephrine renal plasma flow (RPF, Table I) decreased in all dogs but one (dog 25). The mean renal plasma flow at this time was 63 per cent of control values, with a range of 16 to 96 per cent of control levels. Glomerular filtration rate (GFR, Table I) diminished more than 10 per cent in 17 dogs (range of

change, 11 to 70 per cent), remained unchanged in two (dogs 25 and 31) and increased in one (dog 10). For the entire group the mean value of GFR during the infusion of l-norepinephrine was 71 per cent of control levels. There was no correlation between the magnitude of blood pressure elevation and the degree of change in GFR. The correlation between the rate of infusion of l-norepinephrine and the change in GFR was significant ($r = -.446$; $p < .05 > .01$). The filtration fraction (FF, Table I) increased in 11 dogs, decreased in 4 and remained unchanged in 5. On cessation of infusion of l-norepinephrine RPF, GFR, and FF returned towards control values (recovery period, Table I).

Urinary hemoglobin excretion ($U_{Hgb}V$, Table I, Figure 1) increased during the pressor response to l-norepinephrine in 17 studies, decreased in 2 (dogs 9 and 11) and remained unchanged in one (dog 25). The mean excretory rate at this time was 166 per cent of control values with a range of 81 to 316 per cent. These changes in excretion were usually but not invariably accompanied by an increase in the concentration of hemoglobin in plasma. In 17 animals the plasma level of hemoglobin increased 10 to 40 per cent (mean change, 20 per cent); in three (dogs 8, 9 and 25) the plasma level did not change. The rise in plasma concentration may be attributed to the hemoconcentrating effects of l-norepinephrine (17, 18) and, in some instances, to a greater rate of administration than of excretion of hemoglobin.

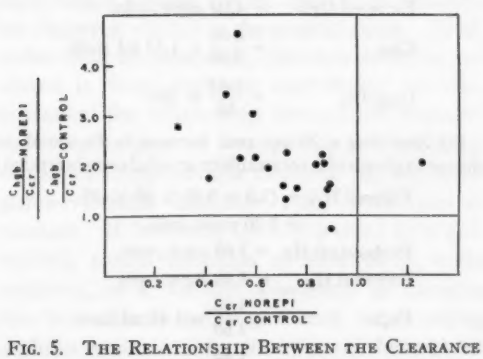

FIG. 5. THE RELATIONSHIP BETWEEN THE CLEARANCE OF CREATININE AND THE HEMOGLOBIN-CREATININE CLEARANCE RATIO DURING THE PRESSOR RESPONSE TO L-NOREPINEPHRINE

The ratio of the infusion to the control values of the hemoglobin-creatinine clearance ratios (C_{Hgb}/C_{Cr}) is plotted against the ratio of the infusion to control values of the clearance of creatinine (C_{Cr}). As C_{Cr} diminished, the clearance of hemoglobin increased relative to creatinine. In one study C_{Hgb}/C_{Cr} increased as C_{Cr} rose.

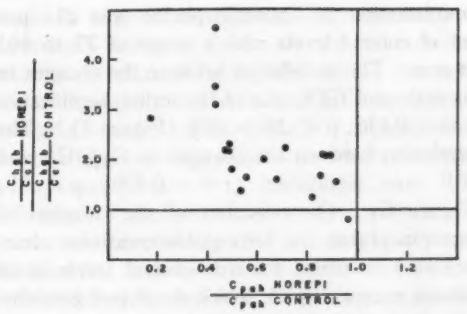

FIG. 6. THE RELATIONSHIP BETWEEN THE CLEARANCE OF PAH AND THE HEMOGLOBIN-CREATININE CLEARANCE RATIO DURING THE PRESSOR RESPONSE TO L-NOREPINEPHRINE

The ratio of the infusion to the control values of the hemoglobin-creatinine clearance ratios (C_{Hgb}/C_{Cr}) is plotted against the ratio of the infusion to the control values of the PAH clearance. As the clearance of PAH diminished, the clearance of hemoglobin increased relative to creatinine.

The renal clearance of hemoglobin (C_{Hgb}, Table I) showed similar directional changes (Figure 2). However, since the concentration of hemoglobin in plasma increased in most studies, the percentage change in hemoglobin clearance was less than that of hemoglobin excretion. C_{Hgb} increased in 14 animals, decreased in 4 (dogs 9, 11, 19 and 25) and remained unchanged in 2 (dogs 14 and 16). This variability may be attributed in part to the magnitude of the reduction in the volume of glomerular filtrate, since the clearance of hemoglobin tended to fall when GFR was reduced more than 50 per cent (Figure 3). The mean C_{Hgb} during the infusion of l-norepinephrine was 137 per cent of control values with a range of 69 to 267 per cent.

On cessation of the infusion, the excretion and clearance of hemoglobin returned towards control values in three studies (dogs 33, 34 and 35) and increased in three (dogs 17, 18 and 25). The increase in dog 25 was spurious since this animal developed gross hematuria during the recovery periods.

The hemoglobin-creatinine clearance ratio (C_{Hgb}/C_{Cr}, Table I) increased in every study but one (dog 25) (Figure 4). The mean value during the

administration of l-norepinephrine was 211 per cent of control levels with a range of 75 to 463 per cent. The correlation between the changes in this ratio and GFR was of borderline significance ($r = -0.436$; $p < .10 > .05$) (Figure 5).[5] The correlation between the changes in C_{Hgb}/C_{Cr} and RPF was significant ($r = -0.590$; $p < .01$) (Figure 6). On cessation of the infusion of l-norepinephrine the hemoglobin-creatinine clearance ratio returned towards control levels in all animals except dog 25, which developed gross hematuria.

DISCUSSION

Hemoglobin molecules circulating in plasma are excreted in the urine at a rate which is determined by the rate of transport of these molecules across the glomerular membrane and by the capacity of the renal tubular cells to abstract this protein from glomerular filtrate during the process of urine formation. The present study suggests that either one or both of these processes concerned in hemoglobin excretion may be modified during the pressor response to l-norepinephrine. The increase in urinary excretion and in the renal clearance of hemoglobin elicited by the infusion of l-norepinephrine may be attributed to either an increase in transglomerular transport of hemoglobin or to a decrease in tubular reabsorption, or to both. Although the precise mechanism of this excretory response was not established, the magnitude of the changes suggests that alterations in tubular reabsorption alone were not responsible. In dogs 12, 13, and 33 the increment in hemoglobin excretion exceeded 2.0 mgm. per minute (by 2.2, 4.62, and 2.83 mgm. per minute, respectively), the approximate maximal rate of tubular reabsorption of hemoglobin in dogs the size of those employed in the present study (10). Hence, the augmented hemoglobin excretion may be attributed, at least in part, to a more rapid rate of transfer of hemoglobin molecules into glomerular filtrate. Whether changes in tubular reabsorption also occurred is not known.

The mechanism of this alteration in transglomerular transport is not clear. Since the concentration of hemoglobin in glomerular filtrate rises in a linear manner as plasma hemoglobin concentration increases (10, 19), the increment in plasma concentration usually elicited in the present study by the infusion of l-norepinephrine undoubtedly contributed to this increase in transport in most studies. However, the increase in plasma level was inconstant (dogs 8 and 9) and was insufficient in most instances to account for the magnitude of the increment in the clearance of hemoglobin and in the hemoglobin-creatinine clearance ratio.[6] Hence other factors appear to have been operative as well to augment the glomerular transport of this protein.

That alterations in glomerular filtration rate may have initiated these changes in transport must

[5] When the one study (dog 10) in which the creatinine clearance increased is omitted from these calculations, the correlation between GFR and C_{Hgb}/C_{Cr} is significant ($r = -0.539$; $p < .02 > .01$).

[6] This view can be illustrated by theoretical calculations of the change in the clearance of hemoglobin and in the hemoglobin-creatinine clearance ratio to be expected by a 20 per cent increase in the plasma hemoglobin concentration, the average increment observed in the present study during the infusion of l-norepinephrine.

(a) Assuming the following values: plasma hemoglobin concentration, 3.0 mgm. per ml.; creatinine clearance, 40 ml. per minute; glomerular permeability to hemoglobin, 5 per cent of the creatinine clearance (18); and $T_{M_{Hgb}}$, 2.0 mgm. per minute:

$$\text{Filtered Hgb} = 3.0 \times 40 \times .05 = 6.00 \text{ mgm./min.}$$

$$\text{Reabsorbed Hgb} = 2.00 \text{ mgm./min.}$$

$$\text{Excreted Hgb} = 4.00 \text{ mgm./min.}$$

$$C_{Hgb} = \frac{4.00}{3.00} = 1.33 \text{ ml./min.}$$

$$C_{Hgb}/C_{Cr} = \frac{1.33}{40} = .033$$

(b) Assuming a 20 per cent increase in P_{Hgb} and no change in glomerular permeability or tubular reabsorption:

$$\text{Filtered } H_{gb} = (3.0 + 0.6) \times 40 \times .05 = 7.20 \text{ mgm./min.}$$

$$\text{Reabsorbed } H_{gb} = 2.00 \text{ mgm./min.}$$

$$\text{Excreted } H_{gb} = 5.20 \text{ mgm./min.}$$

$$C_{Hgb} = \frac{5.20}{3.60} = 1.44 \text{ ml./min.}$$

$$C_{Hgb}/C_{Cr} = \frac{1.44}{40} = 0.36$$

Per cent change in C_{Hgb} = 8 per cent.

Per cent in C_{Hgb}/C_{Cr} = 9 per cent.

These changes are less than those determined experimentally (average experimental change in C_{Hgb}, 37 per cent and in C_{Hgb}/C_{Cr}, 111 per cent). Hence an increase in plasma hemoglobin concentration of 20 per cent cannot alone account for the observed alterations.

be considered in view of the relationship between protein transport and filtration rate as formulated in the theory of molecular sieving. According to this theory, the concentration of protein in capillary filtrates increases as the rate of formation of filtrate diminishes, owing to differences in the diffusion characteristics of protein and water (creatinine) molecules (2–4). Although GFR characteristically fell in the present study, the role of this reduction is obscure. That the excretory response to l-norepinephrine may have been *modified* by changes in GFR is evident in Figure 3, which shows that the typical increase in hemoglobin clearance was abolished by marked decrements of GFR.[7] But whether adjustments of GFR *initiated* the excretory response to l-norepinephrine in accordance with the concept of molecular sieving is not clear. In one study (dog 10) the clearance of hemoglobin increased (both in absolute terms and relative to creatinine) in association with an increase rather than a decrease in GFR. Moreover, the relationship between filtration rate and protein transport is uncertain owing to doubt concerning the mechanism of the reduction of GFR in the dog during the vasoconstrictive response to l-norepinephrine. If, as has been suggested (20), filtration diminishes as a result of selective cessation of glomerular activity in a portion of the nephron population, the theory of molecular sieving as outlined cannot account for the changes in hemoglobin clearance elicited in the present study. Since, under these circumstances, filtration would be unaltered in those nephrons contributing to urine formation, the relationship between the transglomerular transport of hemoglobin and creatinine would not change in these nephrons and the hemoglobin-creatinine clearance ratio would remain constant. If, however, GFR decreases by a generalized, partial reduction of filtration in active nephrons, or if variable alterations of filtration rate in residual, active glomeruli occur during nephron exclusion, the transport of hemoglobin across the glomerular membrane of functioning nephrons might increase relative to creatinine in accordance with the theory of molecular sieving and the clearance of hemoglobin would rise relative to creatinine. Until, however, the precise mechanism of the reduction of GFR is established, the role of these changes in filtration rate cannot be evaluated.

Studies of the effects of plasma volume expansion on proteinuria (5) and of the effect of renin on the excretion of hemoglobin and plasma proteins in rabbits and rats (7–9) have suggested that the transport of protein molecules across the glomerular membrane may be conditioned by the level of intracapillary hydrostatic pressure. Renin, which has hemodynamic effects in the kidney similar to those elicited by l-norepinephrine, is thought to elevate intraglomerular pressure by vasomotor adjustments of the efferent and afferent arterioles (8). This rise in pressure, it has been suggested (8), accelerates the filtration and/or diffusion of protein across the glomerular membrane by mechanisms which are not clear, but which may be related to stretching of the glomerular membrane (5). Whether l-norepinephrine acts in a similar manner is not known. A rise in intraglomerular pressure would be expected to elevate glomerular filtration rate relative to renal plasma flow (an increase in the renal filtration fraction), a functional pattern which is elicited by both renin and l-norepinephrine. But whether this relationship between filtration rate and plasma flow actually reflects adjustments of intraglomerular pressure is not clear (15). Moreover, if nephron exclusion occurs during the administration of l-norepinephrine, a variable and uncertain relation between flow and filtration may exist in active and inactive nephrons and hemodynamic interpretation of the filtration fraction would be impossible. Hence the changes in protein transport elicited in the present study cannot be evaluated in terms of adjustments of intraglomerular pressure. Whether the level of hydrostatic pressure genuinely influences protein transport has not been unequivocally established. The precise role of this factor in the kidney and other sites remains to be determined.

That hemodynamic adjustments within the kidney may have altered the transport of hemoglobin across the glomerular membrane independently of alterations in glomerular filtration rate and intraglomerular pressure is possible. The relationship

[7] The decrease in the clearance of hemoglobin under these circumstances may be attributed to the marked reduction in the volume of glomerular filtrate. Since the clearance of hemoglobin increased relative to creatinine in these instances, the factors tending to augment the transglomerular transfer of hemoglobin appear to have been operative in these as in the other studies.

illustrated in Figure 6 indicates that as renal plasma flow diminished the clearance of hemoglobin increased relative to creatinine. Whether this correlation indicates a causal relationship between renal plasma flow and transglomerular hemoglobin transport is, however, not clearly established. Moreover, the mechanism by which changes in renal plasma flow might condition hemoglobin transport is obscure. An increased rate of transglomerular transport might occur under these conditions as a result of a reduction in the rate and velocity of blood flow through the glomerulus. If these changes in glomerular blood flow accompany a reduction in renal blood flow, the contact between hemoglobin molecules and the glomerular capillary wall may be prolonged, thus allowing more time for the diffusion of these molecules into glomerular filtrate.

This view may explain in part the relatively high values recorded for the glomerular permeability to hemoglobin in both man and dog, since the administration of this protein intravenously is attended by intrarenal vasoconstriction and a reduction of renal blood flow (16). Moreover, the higher values for glomerular permeability elicited when the rate of intravenous administration is increased (19) may be attributable to a greater reduction of blood flow through, and an increased rate of diffusion of hemoglobin from, the glomerulus under these conditions.

SUMMARY

The administration of pressor doses of l-norepinephrine to anesthetized dogs elicited abrupt alterations in the urinary excretion and renal clearance of hemoglobin. When renal vasoconstriction was moderate, the clearance of hemoglobin increased; when renal vasoconstriction was intense and glomerular filtration rate diminished sharply, hemoglobin clearance decreased or remained unchanged, presumably since less filtrate was available for excretion under these conditions. Since the clearance of hemoglobin increased relative to filtration in all studies but one, regardless of the intensity of the vasoconstrictive adjustments, and since this change could not be attributed to a decrease in tubular reabsorption of hemoglobin alone, the administration of l-norepinephrine apparently elicited an acceleration in the transglomerular capillary transport of hemoglobin molecules.

The mechanism of this change in transport was not determined. An increase in plasma hemoglobin concentration usually occurred and contributed to, but was not alone responsible for, the increase in transglomerular hemoglobin transport. The possibility that alterations in renal blood flow played a role was considered. The way in which a reduction in flow might have acted was not established, but it was suggested that if the rate and velocity of glomerular blood flow diminished as renal blood flow decreased, the contact between hemoglobin molecules and the glomerular membrane may have been prolonged, thus allowing more time for the diffusion of those molecules into glomerular filtrate. Although adjustments of glomerular filtration rate and intraglomerular hydrostatic pressure may have contributed to the observed changes in hemoglobin transport, the role of these adjustments could not be evaluated in the present study.

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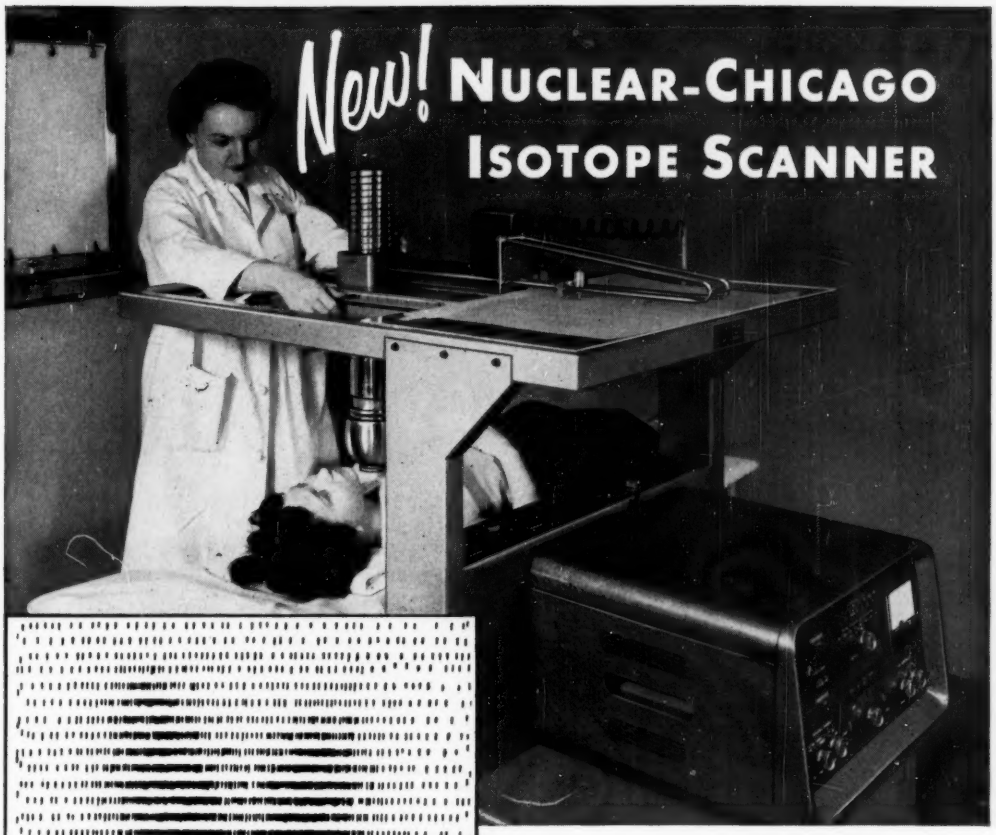

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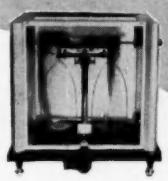

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THE RENAL LESION IN EPIDEMIC HEMORRHAGIC FEVER [1]

By JEAN OLIVER and MURIEL MACDOWELL

(From the Renal Research Unit, Overlook Hospital, Summit, N. J.)

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Structural and functional derangements in the kidney during the course of Epidemic Hemorrhagic Fever run as a continuing thread of cause and effect through a complex of physiological and biochemical disturbances that has perhaps no parallel among the acute infectious diseases. So closely knit are these renal aberrations in the fabric of the clinical syndrome that investigators have found it impractical to disentangle the element of "renal failure" from the related phenomena of shock, hypotension or hypertension and electrolyte or water imbalance, and so simply state as their conclusion that "renal complications" are present in all examples of the disease and at every stage of its progress (1).

The general nature of the renal lesion has been established. In its clinical aspects, it is an example of "acute renal failure" with the typical characteristics of proteinuria, oliguria and late diuresis. From the physiological viewpoint, the investigations of Froeb and McDowell (2) and of Syner and Markels (3) have demonstrated similar disturbances of renal blood flow which occur either as a part, or at times independently, of the general circulatory collapse which so frequently ensues in the early phases of the disease.

The pathological alterations that occur in the kidneys have been described by the Russian investigators who first recognized the disease in eastern Siberia (4),[2] and in later studies by the Japanese (4). More recently, Hullinghorst and Steer (6), Steer (7) and Lukes (8) have reported on material obtained during epidemics among American troops in Korea. Though the resemblance of the histological picture to that which is found in other forms of the acute renal necrosis associated with traumatic and toxic injury has impressed pathologists, certain characteristics of the structural lesions of EHF are so distinctive that in the absence of a demonstrable etiological agent they have become the final criterion for its diagnosis.

A general discussion and detailed analysis of the clinical, physiological and biochemical derangements that occur in EHF have appeared in a Symposium published under the editorial direction of Dr. David P. Earle (9). Against this background an attempt will now be made to integrate the development of the structural aspects of the renal lesion with the progress of its functional disturbances.

MATERIAL AND METHODS

The clinical data and pathological material of this study have been taken from the records of the Commission of Epidemic Diseases and the collections of the Armed Forces Institute of Pathology.

Thirty-nine fatal cases of EHF which occurred during the Spring and Fall epidemics of 1952 and 1953 among American troops in Korea have been examined. An analysis of thirty-four of these appears in the Symposium (9); the case numbers of this earlier study have been retained, and though much of its substance has been incorporated into our report further pertinent information may be derived from this source, in particular from Table VIII, page 636.

The distribution of the cases in regard to the phase of the disease in which the individual died was as follows:

Hypotensive phase:	19
(Primary shock 12)	
(Transition shock 7)	
Oliguric phase:	10
Diuretic phase, 1 to 16 days of diuresis:	9
Convalescence, 149 days from onset:	1
	39

[1] This work was done under the auspices of the Commission on Hemorrhagic Fever, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General of the Army, and in part by a research grant (H-1515-C2) from the National Heart Institute, of the National Institutes of Health, Public Health Service, and the Life Insurance Medical Research Fund.

[2] Though the earlier reported cases of EHF were limited to Manchuria and eastern Siberia, later observations have shown that a very similar disease occurs in the Balkans and Czechoslovakia. The mild "epidemic nephropathy" observed in Finland, Sweden and Norway resembles EHF in some ways, but an identity has not been proved (5).

The method of study was as follows. The autopsy protocols and clinical records were first reviewed. Sections of all the tissues from each autopsy were then examined for whatever relation they might bear to the renal lesion and the sections of kidney studied in detail by the conventional methods of histological examination. In the material of this preliminary survey 10 per cent formalin and Zenker's solution had been the fixative, and hematoxylin and eosin the stain; in subsequent examinations the Masson stain, iron hematoxylin and eosin, Acid Fast Green and certain histochemical procedures were used.

After this examination had established the general nature of the renal lesion, microdissection of the kidney tissue as previously described (10) was done to observe the detail and topographical relations of abnormalities in the nephrons. The dissected nephrons were stained with iron hematoxylin, and camera lucida drawings or photomicrographs were made for permanent record. As will appear in the descriptions of the structural alterations, a continuous comparison was made during the progress of the work between the appearance of the lesions as they were revealed by the two methods of examination.

Selected examples illustrating the course and development of the pathological alterations appear in our illustrations. It is perhaps unnecessary to point out that considerations of expense and space have made necessary a considerable restriction in the presentation of our findings. During the dissection of each case hundreds of nephrons were examined, many were drawn by means of the camera lucida, and a lesser number photographed at magnifications of up to ,200 times. Of the latter only typical examples can be published, and at great reduction; the original of Plate I, for example, is composed of 97 individual 4 by 5 microphotographs which, when assembled to show the complete nephron, cover a space of 4 by 9 feet; much detail is therefore lost in the process of publication.

In the exposition of our findings the nomenclature used by clinical investigators to designate the course of the disease has been adopted. A brief review of the syndrome of EHF may orient the reader for the more detailed description of the physiological, clinical and pathological correlations that are to follow.

THE CLINICAL SYNDROME OF EHF

During all but the first of the five successive phases, Febrile, Hypotensive, Oliguric, Diuretic and Convalescent, into which the clinical course of EHF has been divided, disturbances of renal activity are prominent. The Febrile Phase is characterized by the usual fever, chills, headache and nausea of an acute infectious disease, and ends around the fifth day by defervescence.

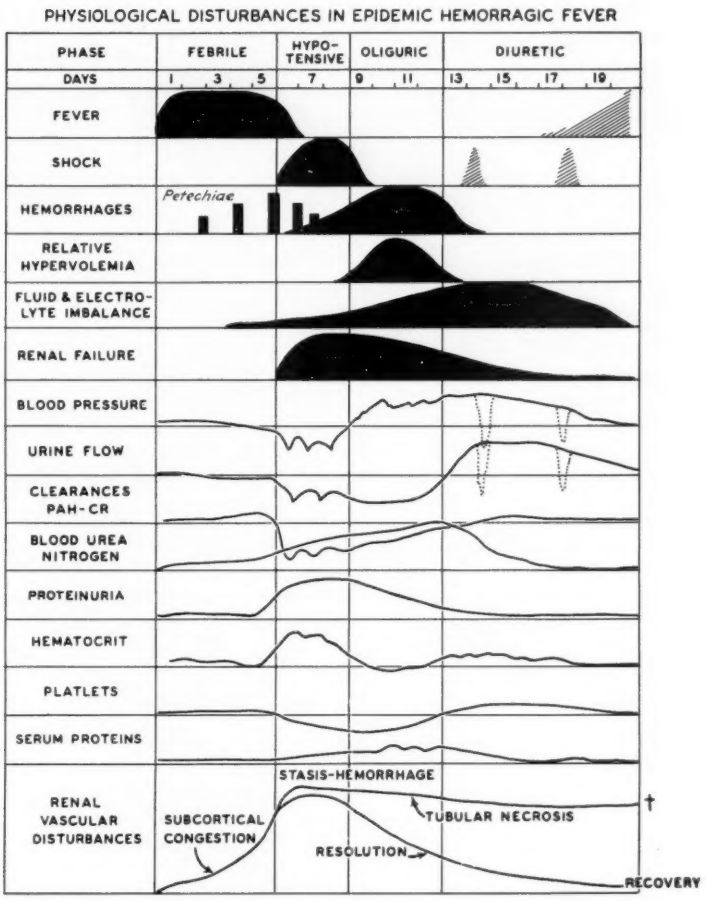
With passage into the Hypotensive Phase, the earlier manifestations of disturbance in the peripheral circulation, such as the intense flush of the face and the injection of the pharynx, become definitive in the appearance of petechiae. Concomitantly, there occurs a rise in the hematocrit which, along with direct determinations of decreased circulating volume, suggests that plasma fluid is leaking through damaged capillaries into tissue spaces. As a result, the blood pressure falls and shock ensues.

These conditions persist during the Hypotensive Phase, in which death from primary shock accounted for one-third of all fatalities. Proteinuria is now massive and the urinary output is irregularly decreased. Blood urea nitrogen rises and, with passage from the Hypotensive Phase, the oliguria, previously irregularly manifested, becomes permanently established to mark the third stage of the disease.

The elevated hematocrit of the Hypotensive Phase has now decreased toward normal, at times abruptly, but more usually requiring one to three days before it becomes stabilized at its original level. During this Transition Period from the Hypotensive to the Oliguric Phase, a circulatory collapse of a nature somewhat different from the "primary shock" of the earlier period may occur; in "transition shock" the observation that total serum proteins do not change as the hematocrit falls, and direct measurements of an increasing circulatory volume, suggest that fluid previously lost to the extravascular spaces is returning.

During the Phase of Established Oliguria which follows, hypertension is commonly observed, but a more remarkable circulatory disturbance during this period is the development in certain cases of a *relative* hypervolemia along with hemorrhagic phenomena during which the complications of pulmonary edema, hemorrhage or convulsions may be fatal.

After 3 to 5 days of oliguria, the Diuretic Phase begins; a daily output as high as 18 L. has been recorded. During this phase abnormalities in electrolyte and water balance may develop and a condition of "limited homeostasis" be established in which secondary shock and pulmonary edema occur. Judging from the evidence of increasing clearances there then follows an improvement in the renal circulation which gradually reaches normal in the following weeks. The BUN has fallen, proteinuria has disappeared, and in the Convalescent Period most patients after two months



TEXT FIG. 1. COURSE OF CLINICAL AND LABORATORY OBSERVATIONS IN SEVERE EPIDEMIC HEMORRHAGIC FEVER

Modified from Sheedy and his associates (1).

can concentrate their urine to a specific gravity of 1.023 or better.

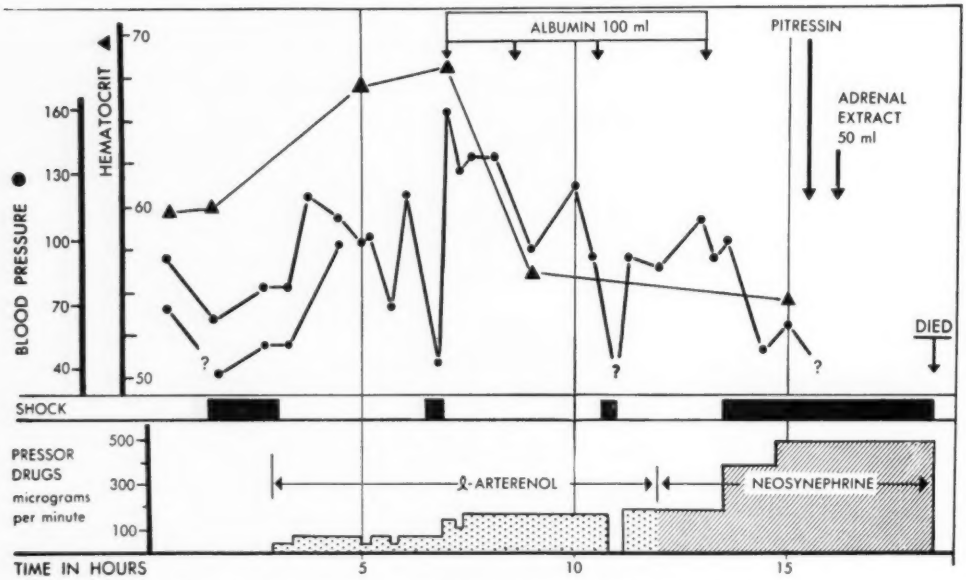
Text Figure 1, which is a modification of Figures 2 and 3 of Sheedy and his co-workers (9), shows the clinical manifestations and laboratory measurements of EHF in graphic form. The lowest level illustrates the occurrence and relative intensity of certain pathological phenomena that were observed in the kidney, and will be explained by our later descriptions.

Although, as previously stated, the renal lesion is to be described as it develops in the consecutive clinical stages of the disease, the procedure of presenting the individual cases has been to arrange them in a series based on the evolution of the pathological lesions. As these lesions are of varied nature, involving vascular, interstitial and parenchymal reactions which may develop at differing rates of intensity, such a series can be only approximate in arrangement.

THE RENAL LESION IN THE FEBRILE AND HYPOTENSIVE PHASE

(The Prehemorrhagic Stage of Subcortical Congestion)

The earliest view of the renal lesion in EHF encountered in this series was obtained in Case



TEXT FIG. 2. CASE 38—DEATH IN PRIMARY SHOCK PHASE OF HEMORRHAGIC FEVER

Note initial response to continuous intravenous pressor therapy but increasing requirements during subsequent course, and death after 18 hours' observation despite 400 cc. concentrated human albumin, Pitressin® and adrenal cortical extract. Note also shock at eleventh hour when L-arterenol infusion infiltrated into subcutaneous tissue. Blood pressure could be measured only by palpatory method after fifth hour. Marked retroperitoneal edema was present at autopsy.

Text Figures 2, 4, 6, 7 and 9 and their legends are from the Symposium on Epidemic Hemorrhagic Fever (9) and are published by permission of author and publisher from the American Journal of Medicine, 1954, **16**, 617.

38, who died on the fourth day of his illness. After three days of severe headache, chills, fever, nausea and vomiting, he entered the hospital with a temperature of 103°, a blood pressure of 100/70 mm. Hg, a 1 + proteinuria and a hematocrit of 59 per cent. Physical examination showed intense injection of the conjunctivae and soft palate, but no petechiae were present. During the examination he suddenly vomited and collapsed; his blood pressure fell to 58/0 mm. Hg. In spite of continuous treatment,[3] including the infusion of 4 units of serum albumin which produced intermittent re-establishment of blood pressure (Text Figure 2), he expired in shock 18 hours after admission. During this period, with a fluid intake of 2864 cc. he passed 415 cc. of urine; the last sample before death showed 2 + protein and the terminal BUN was 42.3 mg. per cent.

At autopsy no petechiae were present. There was a considerable amount of retroperitoneal edema lightly stained with the dye, T-1824, that had been injected for determination of blood volume. The kidneys were not swollen (175 to 200 gm.) and the prosector noted that though the cortex was more sharply differentiated from the medulla than normally, there was not the intense congestive zone which he was accustomed to find as typical of EHF. There were, however, the usual hemorrhages in the right atrium and an extensive hemorrhagic infiltration of the anterior lobe of the pituitary.

On histological examination the glomerular

[3] In this and the cases which follow, only that therapeutic procedure has been given for which there is evidence that it may produce a structural alteration in the kidney, namely, the administration of concentrated human serum albumin (1 unit = 100 cc. 25 per cent salt-poor serum albumin). Many individuals also received whole blood transfusions, glucose and nor-adrenalin infusions and occasionally Dextran. A summary of these procedures may be found elsewhere (9).

tufts were essentially normal; the capillaries contained red blood cells in normal numbers and there was precipitated granular material in Bowman's space. Cross sections of proximal convolutions in the cortex were lined with their typical epithelium which showed some evidence of apical swelling of its cells (Figures 1 and 2); the cytoplasm of these cells was normal. The lumens of cross sections of ascending limbs in the medullary rays and the distal convolutions in the cortex were dilated and appeared empty (Figure 2).

In the subcortical medullary zone were scattered areas of dilatation of intertubular vessels which were crowded with discrete red blood cells (Figure 3). The thin outlines of the intact walls of these vessels were clearly evident and there was no escape of blood into the interstitial tissue (Figure 4). In these areas of congestion the epithelium and basement membranes of the "straight tubules", terminal proximal convolutions and ascending limbs of Henle's loop, though somewhat compressed, were well preserved. Throughout the deeper portions of the medulla were irregularly distributed patches of intertubular edema, but little evidence of congestion and none of hemorrhage (Figure 5). The tubules of the collecting system were normal and contained few casts.

Microdissections of the renal tissue showed essentially normal configurations in the nephrons, except for some slight pressure effect on those terminal segments of the proximal convolutions and ascending limbs of Henle's loop which passed through areas of congestion.

Plate I, A to F, shows a complete nephron stained with iron hematoxylin. Apart from the localized effects just noted, the general configurations of this nephron are so slightly disturbed that it may serve as a control example of the appearance of a normal nephron prepared by our technique for comparison with the damaged specimens that are to follow. In all the preparations, the intense black staining of the proximal convolution is the reaction of the mitochondria which fill the cytoplasm of its epithelial cells in decreasing amount as one departs from the glomerulus; these cellular organelles no longer exist in their original rod-like form but, apparently as a result of the maceration in HCl, are resolved into fine "granules". As in histological preparations stained with iron hematoxylin, the nuclei do not stain heavily but appear as light round spots on the dark background of the cytoplasm, except in the premitotic phases of regenerative proliferation when their excessive and hyperchromatic chromatin makes them clearly visible. It should be recalled that the tubule is being viewed through its entire thickness, 60 to 70 μ in the case of the proximal convolution, so that cellular detail is of necessity somewhat clouded; on the other hand this increase in the absolute amount of tissue under observation at times permits the recognition of cytoplasmic lesions which are not appreciable in thin histological sections. Since a familiarity with the microscopic appearance of dissected nephrons is essential to the recognition and the interpretation of the alterations that are to be seen in the abnormal specimens, a detailed description of Plate I will be given.

The glomerulus, due to the bulk of its tissues, shows little cellular detail; its size and shape are normal, however, and the relation of the more opaque tuft to Bowman's capsule is evident.

The proximal convolution is of normal configuration and diameter, except in its terminal medullary portion, which, passing into an area of congestion in the subcortical outer stripe of the outer zone of the medulla, is somewhat compressed. The cytoplasmic pattern is in general well preserved; the normal mitochondrial gradient is shown by the gradual decrease in the intensity of the reaction to iron hematoxylin, and the clear, vacuolar, round nuclei, more visible as the intensity of the mitochondrial staining lessens, are normally distributed in the tubule wall. At *a* in Plate IB, the medullary segment of the convolution entered an area of intense intertubular congestion which lay just below the cortex. The tubule is definitely narrowed, and although its cells are in general well preserved, slight irregularities are noted toward the end of the segment, and a scattering of intracellular detritus indicates some epithelial damage.

The greater part of the thin portion of Henle's loop, though successfully dissected and mounted, was swept away during the process of staining (Plate ID); a short remnant, somewhat dilated, remains (Plate IE) and passes abruptly into the thick portion of the limb which, continuing to descend, forms the loop and turns towards the cortex. In the area of congestion, lying adjacent

to the compressed terminal segment of the proximal convolution, the cellular pattern of the ascending limb is slightly disturbed and there is some deeply stained intracellular detritus. Above this point and extending high into the cortex the ascending limb is moderately dilated; its cellular components appear essentially normal.

The distal convolution (Plate IF) is much more and very unevenly dilated, so that there is a consequent patchy thinning of its wall which accounts for the irregularity of its staining; there are no coagula or casts in its distended lumen. The juncture of its terminal portion, the connecting tubule, with that of a neighboring nephron is not dilated.

Dissected collecting tubules, from cortex to deep medulla, were entirely normal; those which passed through the areas of congestion were unaffected by the dilatation of the intertubular vessels which surrounded them.

The nephron shown in Plate I was typical of those which were involved in an area of subcortical congestion, and none of these showed more severe alterations. Those which passed outside or between the scattered areas of congestion, at a rough estimate perhaps one-half of all nephrons, showed no visible abnormalities in their structure.

Text Figure 3 is a graphic representation of a renal lobule from this kidney. The nephrons shown are camera lucida tracings of dissected specimens. They and the areas of intertubular congestion are arranged as they were observed to lie in the kidney during the course of dissection and as similar structures and relations appeared in histological sections. For the sake of clarity only a few nephrons are shown and these are widely spaced, but otherwise the figure presents not an interpretive diagram but a reconstruction of actuality.

It is clear that the architecture of the kidney and the topographical relation of its constituent nephrons have not been greatly affected by the vascular disturbance in the subcortical medulla. The dilatation of the distal portions of the nephron lying in the cortex, including ascending limbs of Henle's loop and distal convolutions, is, however, definite if not striking (Figure 2 and Plate IF); this dilatation was associated with a zone of moderate pressure effect from the congestion in the sub-cortical medulla, a relation that will assume greater significance with the progress of the renal lesion.

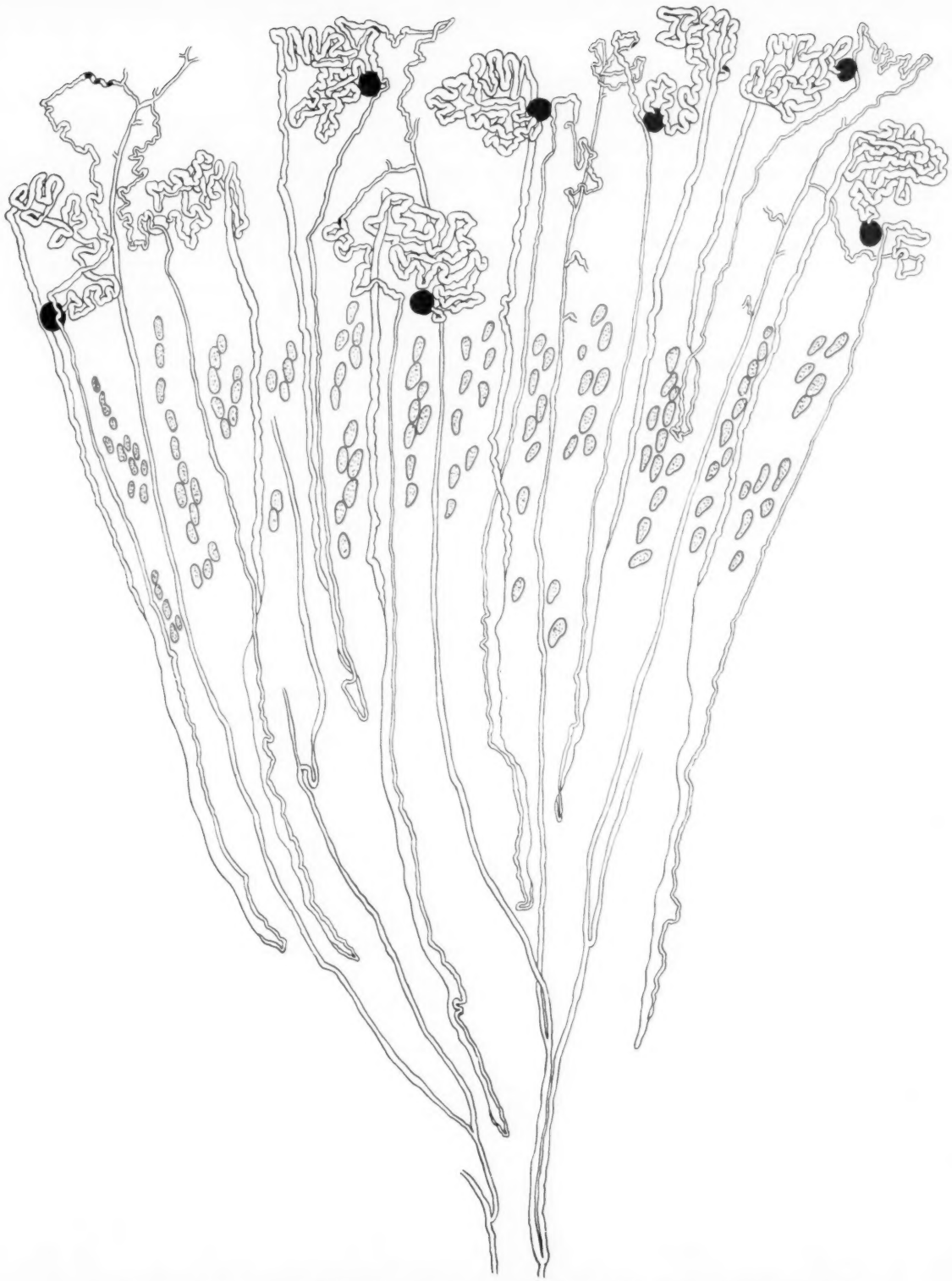
* * * * *

Since the renal lesions become increasingly complex with the progress of the disease, a summary of the structural findings and a discussion of their significance along with a reference to pertinent literature will be given at the end of each clinical period. This procedure may make for reiteration, but will perhaps be less burdensome to the reader than the continuing back reference to minutiae long since described which would be required if critical considerations were postponed to a final conclusion.

The kidney of this individual in whom defervescence was interrupted by sudden death from primary shock may be taken as illustrative of the renal status during the late Febrile Phase at its passage into the Hypotensive Phase. The lesion can be briefly summarized. There is a marked congestive hyperemia localized to the subcortical zone of the medulla, occurring not diffusely but in irregular patchy areas, with no intertubular hemorrhage. There are some slight localized pressure effects of this congestion, both of compression and dilatation, on the tubules of nephrons that by chance pass through it, but no structural evidence of general cellular damage in any of the tubules. The tubular passages are clear; there are few casts.

The correlation of these structural changes with the clinical and physiological data of the period seems reasonably clear; all lines of evidence indicate the beginning of widespread disturbances in the peripheral vascular bed which are characterized first by dilatation and then by increased permeability of capillaries and venules.

In the kidneys, the functional effects of these disturbances are reflected in the clearance examinations of Froeb and McDowell (2) and of Syner and Markels (3), who found not a lessening of renal blood flow, as is observed in classical acute renal failure, but either its full maintenance or actual increase. The histological sections show the distribution of this abundant blood flow in the congestive hyperemia of the subcortical medulla. To a consideration of the reason for this localization we shall return in a later description of the progress of the vascular lesion. Neither histo-



TEXT FIG. 3. A RENAL LOBULE FROM THE EARLY HYPOTENSIVE PHASE OF EPIDEMIC HEMORRHAGIC FEVER—CASE 38

Except for some dilatation of the distal convolutions the topography of the nephrons is normal. There is an irregular intense congestion of the subcortical medulla but no intertubular hemorrhage. Magnification 18 ×.

logical sections nor dissections showed frank structural alterations in the nephrons, a finding which correlates with the clinical observation that there was only a moderate degree of proteinuria and elevation of BUN.

It may be concluded, therefore, from both the structural and functional findings, that the vascular bed of the kidney shares in the general disturbance of the peripheral circulation, the clinical evidences of which were vividly present in the intense flush of the skin of the face and chest and mucous membranes. All these phenomena together constitute the first evidences of generalized capillary damage, and in the case described they terminated abruptly in primary shock when plasma escaped into the extravascular spaces, a phenomenon indicated by the clinical observation of a rising hematocrit and confirmed by the pathological finding of massive retroperitoneal edema. It is noteworthy that the ultimate lesion of vascular damage was as yet not developed in either the skin or the kidney, for neither petechiae were present nor intertubular hemorrhage. This gravest aspect of the vascular lesion was, however, present in two areas, the right auricle and the anterior hypophysis; the small vessels of these regions are apparently peculiarly susceptible since in every case in this series they were the seat of extensive hemorrhage.

* * * * *

The remaining cases of those dying in primary shock may be considered to have definitely entered the Hypotensive Phase, a conclusion supported not only by the evolution of the clinical symptomatology but by the progress of the renal lesion.

The first of these, Case 9, entered hospital with a 3 + proteinuria 3½ days after a typical febrile onset. The next morning the hematocrit rose to 61.9 per cent, the blood pressure fell, and the patient went into shock; in spite of the administration of 1200 cc. of blood and 3 units of serum albumin, he died in shock the morning of the following day. There was no oral intake of fluid, and there was a urinary output of 760 cc.; the terminal temperature was 106°; the BUN on the day before death was 16.8 mg. per cent. At autopsy, marked retroperitoneal edema, hemorrhages in the right auricle and an extensive hemorrhagic infiltration in the anterior pituitary were present. The kidneys were swollen; their medullae are described as "reddish purple".

Histological examination showed an intense, patchy engorgement of the subcortical zone of the medulla. In certain areas the capillaries between the terminal proximal convolutions and ascending limbs of Henle's loop were dilated to the diameter of the adjacent tubules and crowded with red blood cells which in some vessels were discrete and in others packed and fused into a hyaline mass (Figure 6). In spite of this distention the walls of the vessels were not greatly thinned and their intact endothelial cells were plainly visible. The tubules surrounded by these vessels were compressed by the resulting tension and their epithelium showed a moderate degree of protoplasmic disturbance.

Not only were the small vessels of the outer medulla distended with red cells, but the capillaries of the swollen glomerular tufts throughout the cortex were greatly dilated and packed with red blood cells (Figure 7). There was considerable precipitated granular material in Bowman's space. Between the cross sections of the cortical proximal convolutions the intertubular capillaries were not particularly prominent. Both the large veins and the arteries of the corticomedullary junction were, however, engorged with blood.

Whereas the epithelium of the proximal convolutions of the previously described case was essentially normal, there was now present diffusely throughout the cortex a definite swelling of the epithelial cells, and their protoplasm showed irregular variations in density without, however, the occurrence of definite vacuoles. Although the epithelial protoplasm was increased in its granularity, no large, discrete hyaline droplets were present.

* * * * *

In this individual, who died somewhat later in the course of the disease than the previous case, a definite increase in renal structural change is apparent. The subcortical congestion is more intense, though the excess blood is quite clearly contained within the dilated straight vessels of the outer medulla. There is also a marked congestion of glomerular capillaries and somewhat less of the

cortical intertubular network, and both the large arteries and veins are greatly distended. One has the impression, since there was no general visceral congestion observed post mortem to account for the excess blood in the kidney, that dilatation of an atonic intrarenal vascular bed had, by a reduction in peripheral resistance, flooded the entire organ.

The other advance in the development of the renal lesion concerned the epithelium of the proximal convolutions. Throughout their length there was noted a frank protoplasmic disturbance.

* * * * *

Case 14 was a man admitted on the third day of his illness with a temperature of 105° and a blood pressure of 128/70 mm. Hg; on the third day in the hospital it fell suddenly to 90/74. Three units of serum albumin were given. The urine showed a 3 + proteinuria; on an intake of 800 cc. his urinary output was 2280 cc.; the BUN was 24 mg. per cent.

The renal lesion may be briefly summarized as similar to the preceding case, except that the congestion was more closely limited to the subcortical medulla, and the protoplasmic swelling of the cells of the proximal convolutions was more pronounced.

A further development of the disturbance in the cortex of the kidney was noted in Case 20. This man died of primary shock on the seventh day of his illness in spite of the repeated administration of infusions over a period of 3 days of serum albumin to a total amount of 8 units. There was no fluid intake other than by these infusions, and there was a urinary output in 4 days of 1060 cc. His urine was negative for protein for the first 5 days; then a trace appeared increasing in a few hours to 4 +. BUN on entrance was 22.9 mg. per cent, and on the day before death, 85.0 mg. per cent.

Histological examination showed the subcortical congestion and in the cells of the proximal convolutions the protoplasmic disturbance that has previously been described; the latter had advanced to the formation of definite vacuoles. In addition, irregular areas of edema were scattered throughout the cortex, the cross sections of tubule being widely separated by collections of fluid (Figure 8). The cells of the proximal convolutions in the congested subcortical zone were filled with large hyaline droplets.

Case 26 was admitted on the third day of his illness with a temperature of 105°; his urine was free of protein. Shock intervened almost immediately and continued in spite of the administration of 16 units of serum albumin over a period of 3 days. On the fifth day of his illness, the urine showed a 4 + proteinuria. His urinary output during hospitalization was 460 cc.; the BUN rose to 69 mg. per cent; he died in shock on the sixth day of the disease.

At autopsy, massive retroperitoneal edema was noted. The prosector was impressed by the "dark appearance" of the subcortical congestion. Histological examination showed this congestion in the form of dilated but intact intertubular vessels; congestion was not excessive in the cortex. The glomeruli were normal but the capsular spaces contained considerable amounts of precipitated protein. The most pronounced abnormality noted was the marked swelling of the tubular epithelium in the cortical portions of the proximal convolutions. Vacuolization was extreme, large, clear, rounded spaces extending through the entire depth of the epithelial cells (Figure 9). The terminal segments of proximal convolutions that lay in the subcortical zone of dilated intertubular vessels were less swollen; their lumens contained considerable amounts of clear, coagulated protein in which were enclosed bubble-like spaces and many desquamated cells with pyknotic nuclei. The cells which still lined the compressed tubule were filled with hyaline droplets.

The increasing alteration of the epithelium of the proximal convolution described in the preceding three cases appeared at a maximum in an individual (Case C) who, entering the hospital with a temperature of 105.8° on the third day of his illness, went into shock on the following morning. In spite of treatment, including the administration of 3 units of serum albumin and 3 units of Dextran, he succumbed with pulmonary edema on the eighth day. The maximum hematocrit on the first day of shock was 64.2 per cent, falling on the seventh day to 32.9 per cent; in 5 days the intake of fluid was 10,505 cc., the urinary output, 1300 cc. A 4 + proteinuria developed and the BUN rose from 21.4 mg. per

cent on the fourth day reaching 250 mg. per cent on the day preceding death.

At autopsy the kidneys showed a moderate degree of cortico-medullary congestion. Retroperitoneal edema was present.

On histological examination the usual dilation of the intertubular capillaries in the cortico-medullary zone was observed. The most striking alteration was an extreme swelling of the epithelium of the proximal convolutions. The finely granular cytoplasm of the cells appeared as if inflated, resulting in a protrusion of their apices into the lumen of the tubule; the brush border was, however, preserved, as were the nuclei which were displaced towards the base of the cells. This intense swelling of tubular epithelium was sharply limited in its distribution to the proximal convolution, the epithelium of the ascending limbs being essentially normal (Figure 10). It extended into Bowman's space, which was in part obliterated by the protrusion of the foamy cytoplasm of the cells of its parietal layer; the epithelium covering the tuft was not involved (Figure 11). No hyaline droplets were observed in the altered epithelium of the proximal convolutions.

* * * * *

The evolution of the renal lesion as shown by the development of structural alterations in the preceding four cases is evidenced by two phenomena; increase in the intertubular congestion of the cortico-medullary zone, and a swelling of the proximal convolutions. The latter has the histological appearance of an increased hydration of the protoplasm of the epithelial cells which reached the point of vacuolization.

The increase in subcortical congestion took the form of a spread in the extent of involved areas rather than in a progressive dilatation of individual channels. In the less far advanced lesion, the groups of dilated intertubular vessels were widely spaced on a background of less affected medulla and had therefore the normal distribution of the horsetail-like strands of capillaries which result from the subdivision of each of the large, straight efferents from juxta-medullary glomeruli in the lower cortex (11). Originally forming isolated clusters, the addition of more dilated capillary radicles at their periphery resulted in their fusion to a more or less continuous band which in the end separated cortex from medulla. The congested zone thus occupied the entire outer stripe and extended into the inner stripe of the outer zone of the medulla. The altered vascular pattern is therefore that which might result from a continuing flow of blood into abnormal intertubular vessels that had lost their tonicity; the localized distinctiveness of this pattern will be considered later.

Swelling of the tubular epithelium and protoplasmic disturbances in its cytoplasm have not received great attention in considerations of the pathogenesis of acute renal failure and are frequently dismissed under the stock description of "cloudy swelling" with the implication that the importance of this at times dubious histological lesion is not great. Funck-Brentano has corrected this arbitrary view in his thesis on the physiopathological mechanisms of anuria in the acute nephropathies (12). In experiments on rabbits, oliguria and anuria were found to follow the infusion of serum containing 50 per cent glucose, whereas no such effect was produced by infusions of serum containing 9.9 per cent NaCl. Sections of the anuric kidneys showed a marked swelling of the epithelial cells of the proximal convolutions very similar to the illustrations of the swollen epithelia in Figures 10 and 11. He concludes that the swelling is due to cellular hyperhydration which is derived as an effect of alterations in the osmotic pressure of the plasma; this he demonstrates by a corrected freezing point determination. Similar changes in the freezing point of the plasma were noted in human clinical examples of the oliguric phase in acute renal failure. There are no data available on plasma osmolarity in EHF, but the results of Funck-Brentano's experiments in the production of epithelial lesions by modification of osmotic factors and consequent shifts in hydration of cells are remarkably similar to those seen in EHF.

In Case 20 of the preceding group there is noted for the first time in our description of the renal lesion the occurrence of hyaline droplets in the epithelial cells of the proximal convolutions, and these objects will be found in many examples that follow. A consideration of their significance in this report would lead to a considerable digression. We shall therefore only state that in our opinion these droplets do not represent an effect of the

disease processes, though these do modify the conditions of their formation, but rather are the result of a therapeutic procedure, namely, the administration of large amounts of human serum albumin. The hyaline droplets are in other words regarded not as the products of some hypothetical cellular "degeneration" but as droplets of absorbed modified protein similar to those which form in the cells of the proximal convolutions of experimental animals which have been given similarly large injections of homologous plasma proteins (13). Since the droplets are therefore not considered an essential feature of the renal lesion of EHF, the data concerning them will be presented elsewhere in a general discussion of hyaline droplet formation in human renal disease and its significance in the metabolism of the plasma proteins.

* * * * *

The three remaining cases from the Hypotensive Phase of EHF show increasing structural change of the nature so far described and the development of another aspect of the renal lesion that is to assume predominating proportions and result in grave renal damage in later stages of the disease, namely, the ill effects of the vascular disturbance on the tubules of the nephrons.

Case A developed fever and nausea the day after his discharge from the hospital following an acute appendectomy from which he had apparently recovered with an uneventful convalescence. Two days after readmission to the hospital a Douglas' pouch abscess perforated and drained spontaneously; he then went into shock. An intra-abdominal hemorrhage was suspected but none was found on laparotomy. Transferred to the EHF hospital in shock, he received 5 units of albumin and other treatment with little effect, and died on the fifth day. He was anuric during his last hospitalization; his BUN rose to 64 mg. per cent.

At autopsy the kidneys presented the usual subcortical congestion and the anterior lobe of the pituitary was hemorrhagic.

Histological examination showed that in the renal cortex the epithelium of the convoluted tubules was greatly swollen and vacuolated, resembling the alterations illustrated in Figure 9; there were few if any hyaline droplets in their epithelial cells except as the convolutions approached the cortico-medullary boundary. In the area of subcortical congestion the excess blood was still contained within widely dilated capillary spaces. The straight tubules in this region were filled with great numbers of what appeared at first glance to be "hyaline casts"; these also extended upwards in the medullary rays (Figure 12). Collecting tubules in the deeper parts of the medulla and papillae showed only moderate numbers of casts.

The terminal medullary segments of the proximal convolutions which lay in the congested subcortical zone were compressed and crowded together though their lumens were irregularly dilated and filled with hyaline material. These collections on closer examination did not have the appearance of solid casts, the great majority being composed of an albuminous fluid which had, under the influence of the fixative, coagulated in bubble-like configurations containing debris and desquamated cells (Figure 13). The epithelial wall lying contiguous to the entrapped fluid was infiltrated with eosinophilic material; its cells were disarranged, in part desquamated, and showed pyknotic nuclei. The protoplasm of the better preserved cells was crowded with hyaline droplets of varying size.

Similar changes were noted in Case 21, who was admitted with a typical 3-day history of EHF and a temperature of 103°, a blood pressure of 118/58 mm. Hg, and 1 + proteinuria. After an uneventful 24 hours, his hematocrit abruptly rose to 59.0 per cent, and his blood pressure fell to 86/64 mm. Hg with a pulse of 140. In spite of 5 units of serum albumin and other supportive treatment, he died in primary shock on the next day, the sixth day of the disease.

At autopsy there was marked retroperitoneal edema; the kidneys presented the typical appearance of subcortical congestion and there was a gross hemorrhage in the anterior pituitary.

Histological examination showed the usual swelling and vacuolization of the proximal convolutions in the cortex and scattered areas of intertubular edema similar to those illustrated in Figure 8. In the markedly congested subcortical medullary zone the terminal proximal convolutions and ascending limbs were compressed and filled with hyaline material and desquamated cells.

The intact epithelium of proximal convolutions was filled with hyaline droplets; these and the entrapped fluid are shown in the dissected specimens of Plate II. Droplet formation was not limited to this region but extended throughout the entire length of the proximal convolutions up to the glomeruli, so that every cross section of them in the cortex was filled with large Gram positive droplets (Figure 14). The integrity of the cells, even when crowded almost to bursting, was apparently preserved for, when visible among the massed droplets, their nuclei appeared normal (Figure 15).

Essentially similar alterations were observed in the kidneys of Case 8. This man was admitted with a typical 5-day history and a temperature of 103°. On the sixth day he went into shock and in spite of treatment, including the administration of 4 units of albumin in 24 hours, he died on the next day. His urinary output was 1410 cc.; there was a 3 + proteinuria.

At autopsy the usual subcortical congestion and pituitary hemorrhage were present. The histological appearances were very similar to those described in the previous case, hyaline droplets filling the cells in cross sections of proximal convolution throughout the cortex as well as in the terminal segments where entrapped albuminous material was concentrated.

* * * * *

A résumé may now be given of the development of the renal lesion as it is seen during the first week of EHF, a period including the Febrile and the Hypotensive Phases of the disease, in individuals where its further evolution was arrested by death from circulatory collapse. Combining the physiological, clinical and pathological data, the following would appear to be the course of events.

In the Febrile Phase, which initiates the clinical syndrome, the physiological evidence of high clearances indicates that renal blood flow is either normal or increased. In the transition from the Febrile to the Hypotensive Phase, various clinical phenomena appear which indicate widespread damage to small vessels, capillaries and venules; the intense and increasing flush of the skin and mucous membranes and, more certainly, widely dilated nail bed capillaries detectable by microscopic examination (14) are indications of loss of arteriolar tone and vasodilatation. It is noteworthy that skin hemorrhages in the form of petechiae develop somewhat later. A rising hematocrit without corresponding increase in serum protein concentration and the post-mortem finding of T-1824 which has escaped into massive retroperitoneal edema are conclusive demonstrations that plasma is now leaking from the damaged vessels. The abrupt occurrence of a heavy proteinuria, which in the Febrile Phase had been insignificant or absent, shows that the renal capillaries as well are involved in this general vascular lesion.

Renal blood flow now decreases as indicated by a depression of clearances (2, 3); this may occur independently of general circulatory collapse and is exaggerated when primary shock intervenes, as it did in all the cases of this series. The result of this decreased flow through the kidney is, however, quite different in case of EHF than it is in the individual with normal renal vessels who suffers a similar circulatory depression as a result, say, of surgical shock. In both instances there is one factor in common—the distinctive anatomical pattern of the vascular bed in the subcortical zone of the medulla; in the kidney of EHF, however, this peculiar vascular bed is abnormal since the vessels which compose it are both atonic and permeable.

The vascularization of the medulla has been the object of extensive anatomical and functional study in recent years. The older descriptions of the *arteria rectae* which supply it from efferents of glomeruli in the juxta-medullary zone of the cortex have been amplified by the studies of Trueta, Barclay, Daniel, Franklin, and Prichard (11), which demonstrate their relatively large diameter. In extensive anatomical and physiological experiments, Block, Wakim, and Mann (15) have revealed the complexity of the reactions that occur in the subcortical zone to various abnormal situations, in particular those of shock and related conditions. Barrie, Klebanoff and Cates have described this vascular bed most aptly as the "arcuate sponge" (16) and have pointed out the possibility of free communication by sinusoids between the arcuate arteries, the medullary network and the veins.

Although there have been some differences in

interpretations of the functional effects of these anatomical peculiarities, our examination of the disturbed renal circulation in the acute renal failure associated with traumatic and toxic injury (10) agrees in both its experimental and pathological aspects with the more detailed and exact studies of Block,Wakim, and Mann (15). These investigators point out three constantly occurring phenomena in their varied experiments in which the renal circulation was disturbed by electrical stimulation of the renal nerves, sudden clamping of the renal artery, injection of substances such as epinephrine, and the reduction by hemorrhage of the mean arterial pressure: 1) when renal vasoconstriction operates in a renal vascular disturbance it appears to be limited to the cortex alone; 2) under conditions of sharply decreased blood pressure and flow in the kidney the medulla may become congested even though blood is not flowing through it; such an appearance, therefore, does not of necessity indicate an actual "shunting" of the renal circulation past the cortex through the medulla into the renal veins; and 3) in all situations, as the blood flow fails, it is best preserved in the cortico-medullary zone of the kidney. They further point out that from the anatomical standpoint two paths are present for the flow of blood into the medulla—the long *arteriolae rectae* and the general network of peritubular capillaries which supply the cortico-medullary region. When renal blood flow is reduced, most of the flow from the cortex into the medulla is through the peritubular network into the cortico-medullary region with a corresponding decrease through the longer *arteriolae rectae* which pass to the deeper medulla. Blood flow may, therefore, continue *into* but not *through* the cortico-medullary region under circumstances in which flow is reduced to a minimum elsewhere in the kidney.

If, in the light of these considerations, one considers the situation in EHF in which after the increased influx of blood of the Febrile Phase, a reduction of flow ensues in a renal vascular bed which is atonic, dilated and permeable to the escape of plasma, it is evident how and why the intertubular vessels of the "medullary sponge" become so intensely engorged with blood in spite of the decrease in RBF which characterizes the Hypotensive Phase. As is illustrated in Figure 6 the contents of the subcortical capillaries become an almost solid mass of entrapped red blood cells. Plasma which has escaped through the abnormally permeable vessel walls is found in areas of intertubular edema which may extend into the medulla (Figure 5) and, by means of medullary rays, into the cortex (Figure 8).

The effect of these circulatory disturbances on the nephrons is already apparent in the studies of individuals who died in primary shock. In the earliest example (Case 38, Figures 1 and 2) they were so minimal as to be of doubtful significance as a factor in the renal lesion. If death from circulatory collapse had not occurred, it is reasonable to suppose that a return of renal blood flow would have resulted in complete restitution of a normal renal status. In most of the examples of the Hypotensive Phase described, however, there was definite and increasing evidence of damage to nephrons, so that here again the structural changes and the physiological aspects of the renal lesion correlate with the clinical indications of a beginning "renal failure" that were shown in the rise of BUN.

The earliest tubular alterations to develop, present indeed in slight degree in Case 38, were swelling and subsequent vacuolization of both the cortical and medullary portions of the proximal convolutions. Except for the increase in volume of the renal cells and a decrease in the density of their cytoplasm which reached its maximum in Case C (Figures 10 and 11), there was no great alteration of their configuration; their nuclei appeared normal. One might suppose, therefore, that here again the renal lesion is reversible.

The similarity in appearance of the swollen epithelium of the Hypotensive Phase to the changes produced by Funck-Brentano by experimentally altering the osmolarity of the plasma has been noted. Whereas in his experiments hyperhydration of the epithelium may be considered the chief factor in the cause of oliguria, acting either directly by luminal compression or indirectly through a modification of renal blood flow from increased intrarenal tension, the relations are less simple in the case of EHF. Decreased urine flow was irregularly observed during the Hypotensive Phase but not the definitive oliguria to be described later, so that incidental factors, such as variations in cardiac output, fall in blood pressure, decreases in renal blood flow and consequent glomerular fil-

tration would seem to be alternative explanations for the variation in renal output in the earlier phases of the disease.

If the effects of cellular swelling and the pressure of engorged intertubular capillaries had not greatly interfered with urine flow through the lumen of the tubule, they nevertheless had produced their deleterious effect on the integrity of the nephrons. This was apparent in the irregular compression, dilatation and entrapment of proteinaceous urine and desquamated epithelial cells in the terminal medullary segments of the proximal convolutions, as illustrated in Figure 13 and Plate II.

In EHF, these tubular changes in the subcortical zone are the first evidences of a destructive damage to the nephrons which increases to irreversible and irreparable alterations in later stages of the renal lesion.

THE RENAL LESION IN THE PERIOD OF TRANSITION

(The Incidence of Intertubular Hemorrhage)

The transition from the Hypotensive Phase to that of Oliguria is characterized by physiological and clinical phenomena which indicate that escaped plasma is returning to the circulation: the hematocrit falls, retroperitoneal edema decreases and direct measurements of plasma volume are higher. In spite of these evidences of circulatory recovery, fatal "transition shock" may occur, apparently due to arteriolar dysfunction (9, p. 695). Eight fatalities occurring under these circumstances appear in this series; all of these showed the development of a structural alteration previously unencountered.

This characteristic lesion of the Transition Period was the appearance of hemorrhage in the congested subcortical zone. Whereas in the previous Hypotensive Phase the excess of blood was contained within greatly dilated intact intertubular vessels (Figures 4 and 6), foci of exudative hemorrhage were now seen scattered widespread through the area of congestion.

Case 39 was admitted on the fourth day of his illness with a temperature of 101°, a blood pressure of 100/80 mm. Hg and a 4 + proteinuria. He went into shock shortly after admission and remained in circulatory collapse, with remissions, in spite of 9 units of serum albumin and other treatment until his death on the seventh day. On the fifth day, his hematocrit was 49.5 per cent. His urinary output was 150 cc. the day before death and the BUN was 50 mg. per cent. Certain details of his clinical course are shown in Text Figure 4.

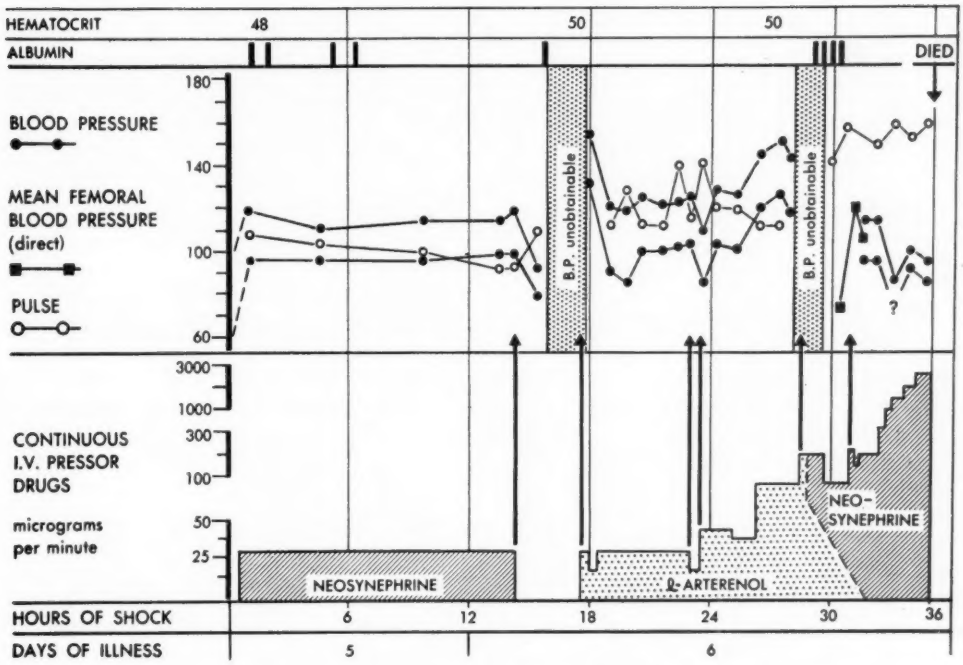
At autopsy there was only a moderate retroperitoneal edema; the kidneys showed the typical congestion of the cortico-medullary zone, and the usual extensive hemorrhage was present in the anterior pituitary.

The general condition of the kidneys was an exaggeration of what has been observed in the more severe cases during the Hypotensive Phase. Swelling and epithelial alteration of the proximal convolutions and scattered focal areas of intertubular edema were present in the cortex (Figure 16); entrapment of albuminous fluid and desquamated epithelial cells was seen in the straight tubules of the subcortical zone of congestion (Figure 17), which, along with irregular areas of intertubular edema, extended into the deeper medulla reaching the papillae. The ducts of Bellini were filled with renal failure casts (Figure 18).

In the subcortical zone of congestion, many capillaries, though packed with red blood cells, were intact; though the outlines of the vessels were still visible, the intertubular spaces between certain tubules contained free red blood cells.

Three other cases, 8, 4 and 27, were so similar in their clinical behavior and in the nature of the pathological lesions found at autopsy that a description of them would be needlessly reiterative. All showed a somewhat higher elevation of BUN; on admission the BUN figures were 27, 49 and 12 mg. per cent, respectively, and on the day before death, 117, 117 and 122 mg. per cent. Proteinuria showed a similar increase from 1 + on admission to 4 + on the day of death, which occurred on the ninth day of illness in the first case and the eighth day in the other two.

Case 28, who died of transition shock on the tenth day, illustrates the acme of intensity in the renal lesion present in this series. He was admitted on the fifth day of his illness with many petechiae and a 3 + proteinuria. He immediately went into shock. In spite of temporary improvement under the usual treatment, which included the administration of 16 units of concentrated se-



TEXT FIG. 4. CASE 39— EXAMPLE OF TRANSITION SHOCK IN HEMORRHAGIC FEVER

Note the dependency of blood pressure on pressor therapy and the relative ineffectiveness of serum albumin therapy despite minimal increase in hematocrit.

rum albumin in 72 hours, he passed from one episode of shock to another, 14 in all, to death on the tenth day. The hematocrit was 53.5 per cent on the first day of hospitalization, and 51.3 per cent on the day before death. A urinary output of 200 cc. was noted on the first day and "less than 50 cc." in the remaining period. Blood urea nitrogen was 58.5 mg. per cent on the day preceding death.

At autopsy only a moderate retroperitoneal edema was observed; the kidney showed extreme subcortical congestion and there was a hemorrhage in the anterior pituitary.

Histological examination showed the subcortical zone to be the seat of widespread infiltrative hemorrhage; the intertubular capillaries, which in examples of earlier stages of the disease were widely distended but whose endothelium was still intact, were now no longer visible as distinct channels. The fibrillar remnants of their walls, however, could be seen scattered through the irregular spaces which, densely packed with red blood cells, isolated each tubular cross section from its neighbor. The enclosed tubules were compressed and distorted and their epithelium necrotic and desquamated so that the characteristic cellular pattern of the various tubular segments was lost (Figure 19).

This intense vascular disturbance separated the cortex by a broad band from the deeper medulla; it was not, however, confined to the subcortical zone. Extending into the cortex with decreasing intensity as the capsule was approached were extensions of the same extreme congestion and frank intertubular hemorrhage (Figure 20). In the areas free of congestion and hemorrhage, the epithelial cells of the proximal convolutions showed the usual swelling and vacuolization of the earlier periods; in tubules surrounded by infiltrating hemorrhage, necrosis of portions of the entire wall including the basement membrane was apparent (Figure 21). The intact epithelial cells of the proximal convolutions were crowded with great numbers of hyaline droplets, both in the

cortical convolutions and in those few terminal straight segments in the subcortical zone which were better preserved.

Congestion, hemorrhage and tubular necrosis also extended deep through the medulla into the papillae. Straight tubules, including collecting ducts, were thus isolated and compressed; their epithelium and even the basement membranes were necrotic over great stretches of their extent (Figure 22). The large ducts of Bellini were surrounded by widely dilated vascular spaces and scattered areas of hemorrhage, and contained renal failure casts (Figure 23).

Dissection of nephrons showed the nature and widespread distribution of the tubular damage, and in particular its topographic relation to the subcortical zone of intertubular hemorrhage.

In Plate III, A to D, all that remained of a proximal convolution is shown; the degree and extent of irregularly scattered tubular disruption as observed when a continuous tubule is examined, rather than in the interrupted cross sections of a histological section, are apparent. Beginning at the origin of the convolution, its cellular pattern is markedly disturbed as can be appreciated by comparison with the more normal convolution of Plate I; nuclei are obscured in the disintegrating cytoplasm of the tubular epithelium, which forms irregular masses of deeply stained material alternating with areas of more lightly stained disrupted tubular wall. The lesion increases in intensity (Plate III, B and C) as the straight segment leaves the cortex and enters the subcortical zone.

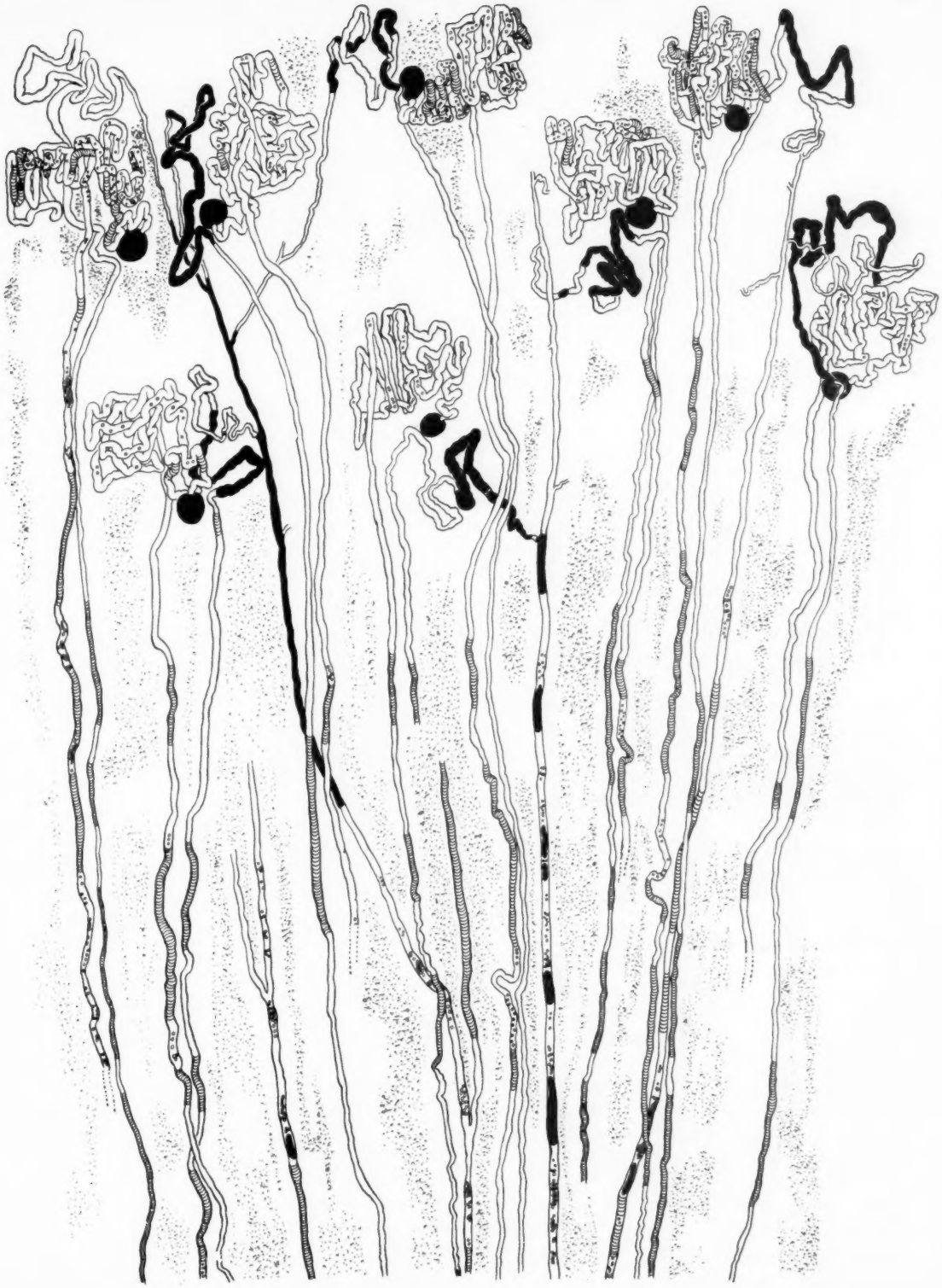
Here the tubule thins to a bare basement membrane which ultimately disappears as its course is interrupted by complete disintegration (Plate III D). An exact determination of the percentage of proximal convolutions thus physically interrupted was impossible since the lesion consists of a gradual attenuation of the tubule in which a few fibrillar remnants indicative of its former course remain. Such remnants are so fragile that they cannot be dissected, and though in a sense they might be considered still existent, they certainly can have none of the functional attributes of a tubule, even the mechanical effect of constituting a confining channel for fluid. Regarded from this simplest of tubular functions, only a few intact terminal proximal convolutions could be found in the zone of hemorrhage.

As previously mentioned, the hemorrhagic area formed an almost continuous band between the cortex and deeper medulla, and extended to the papillae by irregular infiltrations. Within these extensions of the hemorrhage all tubules showed in varying degree the deleterious effect of pressure and anoxia. In Plate IV a fairly intact loop of Henle lying within the hemorrhagic zone is shown, throughout the course of which irregular stretches of damages are evidenced by cellular detritus and the obliteration of nuclear detail; at the bend of the loop the tubule is filled with a dense coagulum which stains black.

Dissected collecting tubules passing through the zone of hemorrhage, though they had preserved their external configuration, were in great part entirely necrotic, showing nothing of the distinctive clear cellular pattern which characterizes these structures; their luminal space was filled with debris and densely black stained casts (Plate V).

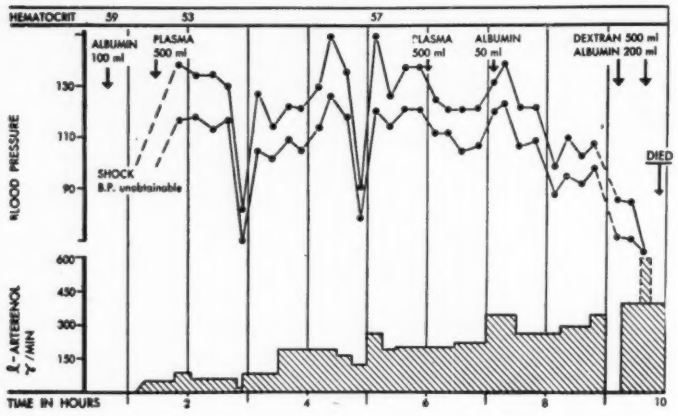
Returning to a description of the cortex in which lay portions of nephrons which, in relation to the direction of urine flow, lay both before and beyond the interrupting band of subcortical hemorrhage, it was evident that the tubules distal to the zone were not only much better preserved than the severely damaged terminal proximal convolutions which lay in it, but in general were less severely affected than the first portions of the cortical proximal convolution which led directly from the glomeruli.

In Plate VI, the origin of a collecting duct is shown with four ascending limbs which, passing into distal convolutions, join by way of connecting tubules to form the peripheral origin of the collecting system. All these structures lay in the outer cortex beneath the capsule and were not involved by the irregularly scattered hemorrhagic infiltration that extended from the subcortical zone. It will be noted that all these tubules are, compared to the severe damage illustrated in the cortical portions of proximal convolutions (Plate III), relatively well preserved. There is, therefore, as in all examples of acute tubular necrosis and renal failure, no preferential damage to the "lower nephron" but rather the reverse. The normal cellular pattern of the ascending limbs that lie in the cortex is visible although some are dilated and contain coagulated material and debris.



TEXT FIG. 5. A RENAL LOBULE FROM CASE 28 WHO DIED OF TRANSITION SHOCK ON THE TENTH DAY

Note the extensive subcortical intertubular hemorrhage with extension into the cortex. Necrosis of tubules within the involved areas. Heavy black, glomeruli and casts within tubules; cross-hatching, necrosis of tubules; small circles within tubules, desquamated cells and debris. Many terminal proximal convolutions end by disruption in the zone of hemorrhage. Magnification 18×.



TEXT FIG. 6. CASE 42—EXAMPLE OF FATALITY IN SEVERE PRIMARY SHOCK IN HEMORRHAGIC FEVER DESPITE CONTINUOUS L-ARTERENOL INFUSION

This patient was in severe shock when admitted and blood pressure was unobtainable until given both plasma volume expander and an infusion containing a large amount of L-arterenol. Accidental stopping or slowing of the infusion resulted in marked decrease in blood pressure. The severe shock that followed the second episode did not respond to further plasma volume expanders or heroic pressor therapy.

The distal convolutions are also dilated, and though cellular detail is obscured by the intense black staining of the solid coagulum that distends their lumens, occasionally a flattened but intact epithelium is visible. The connecting tubules are not dilated and in most instances are free of black stained coagula; this dense material appears again in the peripheral collecting tubules and, obscuring cellular detail, fills the remaining portion of the collecting duct.

Text Figure 5 shows a reconstruction of a lobule from camera lucida drawings of dissected nephrons; it may be compared with Text Figure 3, a similar reconstruction of the earlier stage which showed so little departure from normal topographical relations. The important features now noted are: 1) the presence of an irregular band of hemorrhage separating cortex from medulla; 2) the extension of the congestion and hemorrhage to the papillae on the one hand and, in diminishing scattered areas, to the surface of the kidney on the other; 3) the interruption by necrosis of the majority of all the tubules, terminal medullary segments of proximal convolutions, descending and ascending limbs—both broad and thin—and collecting tubules, which pass through the zone of hemorrhage; and 4) the dilatation with coagulated proteinaceous material of those tubules which lie in the cortex distal to the zone of hemorrhage.

The clinical course of Case 42 is shown in Text Figure 6; admitted in a state of shock on the fifth day of illness, he died in 10 hours in spite of treatment that included the administration of 3½ units of serum albumin and 500 cc. Dextran; 400 cc. of urine were passed. At autopsy a moderate retroperitoneal edema was present as well as the usual subcortical congestion in the kidney and hemorrhage in the pituitary.

Histological examination showed an intense hemorrhagic infiltration throughout the corticomedullary zone. The outlines of the intertubular capillaries could not be made out, red blood cells filling the spaces between the cross sections of tubules in densely packed masses. Congestion and hemorrhage extended deep into the medulla and also into the cortex. Many of the proximal convolutions in the cortex were filled with hyaline droplets; the nuclei of the cells containing them were well preserved. In the areas of subcortical intertubular hemorrhage the tubules were compressed and distorted and their epithelial walls destroyed.

The two remaining cases, which showed massive intertubular hemorrhage in the congested cortico-medullary zone, and which were considered clinically to have died in the late Hypotensive Phase or in the Transition Period of shock, may be briefly mentioned, as the renal lesions were essentially similar to those which have been described. Case 17 survived for 10 days, the last 5 days of which he was in more or less continuous shock in spite of intensive treatment which included the administration of 12½ units of serum albumin during the last 4 days. During this period he passed 190 cc. of urine; it is of interest that his urine had been free of protein during the Febrile Phase until the fifth day, when, with the onset of the Hypotensive Phase, it suddenly showed a 4 + proteinuria.

Case 1 was admitted on the fifth day of illness with a blood pressure of 90/60 mm. Hg, and died the next morning in shock. Two units of serum albumin were given; with a total fluid intake of 1500 cc., his urinary output was 390 cc.

The structural changes were essentially similar in these two cases. Intense subcortical intertubular hemorrhage was present with extension into the papillae and less to the cortex, with tubular destruction not only in the medullary zone but scattered irregularly throughout the cortex and deep medulla. In the individual who had received 12½ units of human serum albumin, the cells of the better preserved convoluted tubules were filled with droplets; these were present but sparse in the other, who had been given 2 units.

* * * * *

Since the period covered by the group of cases just described is by definition transitional from the Hypotensive to the Oliguric Phase, a discussion of the structural findings can be postponed until the full development of the renal lesion in the next period has been given. An important correlation between clinical and pathological aspects should be noted in passing; as the clinical phenomena required the special recognition of an interim between two major phases in the term Transition Period, so the finding of a distinctive structural element, intertubular hemorrhage, sets this period off pathologically in the evolution of renal lesion. There has been no need for such a subdivision in descriptions of general Acute Renal Failure, for no such structural distinction in the form of subcortical hemorrhage occurs.

As will become apparent when the functional effects of this new development are described, its importance in the ultimate fate of the kidney as a collocation of nephrons is critically determinative.

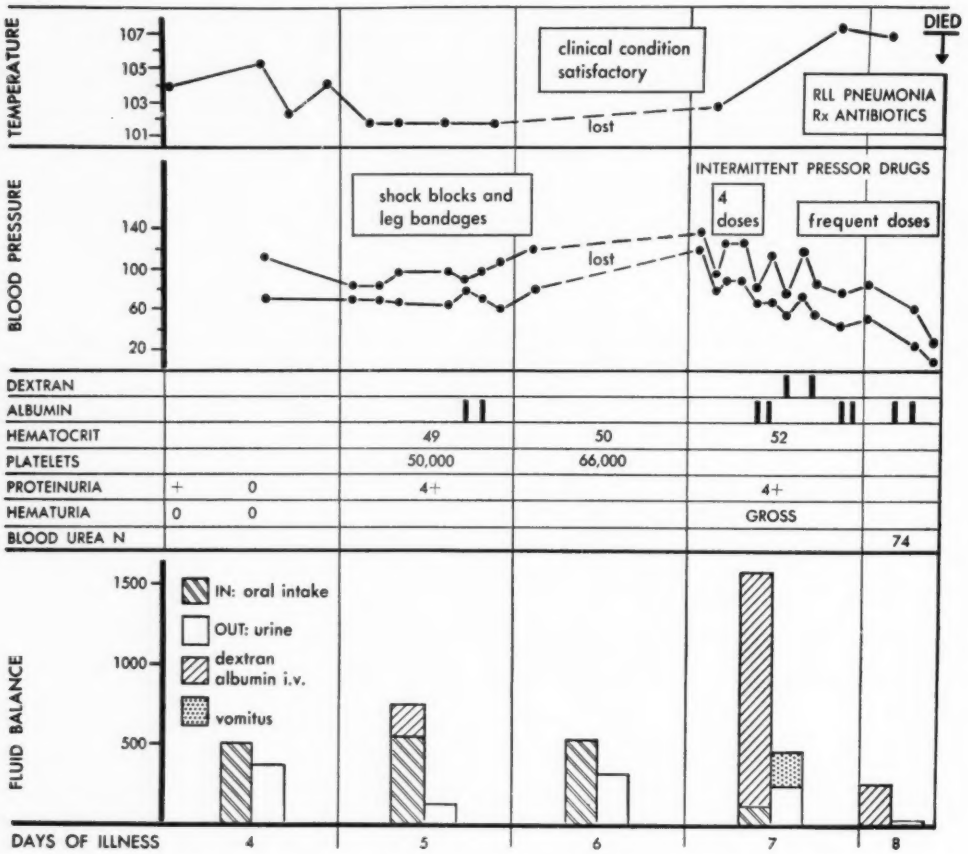
THE RENAL LESION IN THE PHASE OF ESTABLISHED OLIGURIA

(Infiltrative Intertubular Hemorrhage and Stasis; Tubular Necrosis and Intrarenal Obstruction)

Intertubular hemorrhage resulting from increasing congestion that proceeds to ultimate stasis in damaged terminal vessels is not, by the nature of its origin, a sudden event but a gradual infiltration that develops at varying rates depending on the severity of both congestion and vascular damage. It is not surprising, therefore, to find its structural and functional effects spread over three phases of the renal lesion, the late Hypotensive, the Transition Period and the Oliguric Phase.

Ten cases, each of whom died on or between the eighth to eleventh days, presented the clinical characteristics of an established oliguria and showed the full development of the renal transformation of EHF. The physiological disturbances, in particular the detail of fluid balance, of a typical example, Case 23, are shown in Text Figure 7. All cases showed the lesions which have been noted above in our description of those who died in late primary or transition shock, *i.e.*, a subcortical zone of intertubular congestion and hemorrhage with extension of both to the deep medulla and in lesser degree to the cortex, with tubular disruption of those portions of nephrons which were included in the affected areas.

All ten cases showed in addition, however, an added structural change which characterized the Phase of Established Oliguria pathologically, namely, a marked dilatation of the cortical tubules of the nephrons. This is illustrated in Case 31, an individual who entered hospital on the fourth day of his illness, went through the Hypotensive Phase with shock, for which he received 2 units of albumin, and passed into the Oliguric Phase in which he died on the eleventh day with a BUN of 184 mg. per cent. His fluid intake during the last five



TEXT FIG. 7. CASE 23—EXAMPLE OF DEATH IN SECONDARY SHOCK DURING OLIGURIC PHASE OF HEMORRHAGIC FEVER

Primary shock on fifth day during Hypotensive Phase was mild. Subsequent slight increase of hematocrit was presumably due to dehydration rather than to continued capillary leakage of plasma. On the seventh day the patient developed right lower lobe pneumonia and hyperpyrexia that precipitated shock not responsive to therapy. At autopsy characteristic findings of hemorrhagic fever were present, although there was no retroperitoneal edema. In addition, a patchy pneumonia chiefly in the right lower lobe was noted.

days was 4184 cc. and his urinary output was 360 cc.

Histological sections showed a wide dilatation of all tubules in the cortex. In the case of the dilated proximal convolutions, the distention reached Bowman's space with a resulting compression of the glomerular tuft (Figure 24). Not only those portions of the nephrons lying proximal to the zone of medullary hemorrhage were distended but the cortical ascending limbs and distal convolutions, the latter containing cellular debris, were also dilated. The relation of this tubular dilatation to the compression of the subcortical zone of hemorrhage is shown in Figure 25.

The effects of the dilatation of all the cortical portions of the nephron are shown in dissected specimens in Plate VII, A to D. The wide lumen of the proximal convolution is clearly apparent throughout its entire length, as can be seen by comparison with Plate I; the stretching of the epithelial wall increases the space between the nuclei, and so exaggerates the cellular pattern. The epithelial cells are, however, fairly well preserved. As its terminal segment approaches and enters the

subcortical region of congestion it narrows to one-third its previous diameter and is lost in the intertubular hemorrhage. A portion of ascending limb and the distal convolution from the cortex, which was originally entwined with the dilated proximal, and which was therefore known to be the continuation of the same nephron, show an equal dilatation; there is some dark stained material in the distal convolution.

In Case 25 there was more than the usual cellular damage in proximal convolutions, and evidences of beginning repair were visible both in regenerative mitotic proliferation of epithelium (Figure 26) and in the ingrowth of interstitial granulation tissue into the disrupted tubule lumens. Case 41 showed somewhat less dilatation of the lumens of the cortical tubules, and scattered areas of intertubular edema (Figure 27). The remaining cases showed the general picture of cortico-medullary hemorrhage and dilatation of cortical tubules above it, but added nothing further towards the elucidation of the renal lesion.

* * * * *

It is in the Phase of Established Oliguria that the structural and functional lesions of EHF reach the height of their evolution. When the structural changes in the nephron are thus fully established they appear to constitute an irreversible status, for though a "kidney" of sorts might evolve from those nephrons which by the fortunate circumstance of their location had escaped the effects of congestion and hemorrhage, the reconstitution of nephrons whose middle half has been destroyed would seem impossible. We shall see in later descriptions that the attempt is made, but the appropriate anastomosis of four frayed ends of fragile tubule lying loose in a mass of interstitial hemorrhage under considerable pressure is on the sheer basis of probability unlikely, even when the potentialities of repair are present. It would seem, therefore, that the renal lesions we have seen in the fatal cases of established oliguria could not have existed in similar quantitative relations in the great majority of those suffering from EHF, for they survived and went on to eventual recovery.

In the earlier stages of the syndrome tubular damage was evident in the general swelling and vacuolization of the epithelium of the proximal convolution, whereas in the Transition Period and Oliguric Phase the tubular alteration consists of localized physical disruption; the two lesions are therefore quite different in their structural aspect. Though the early epithelial changes are of the sort that have been classically derived from "toxic action," there has been no demonstration of a toxin in EHF other than the hypothetical substance which acts on the small blood vessels. Disturbances of circulation, however, are clearly visible at this time, so that it seems reasonable to accept them as the cause of the early epithelial damage.

Structural interruption of the nephrons involves proximal convolutions, ascending and descending limbs of Henle's loops and collecting tubules indiscriminately. The damage is confined to the areas of congestion and hemorrhage, and so lies chiefly in the medulla, but is also present extensively throughout the cortex when the circulatory disturbance has spread to this area. Here again, it would be difficult to explain this random distribution in terms of the direct action on the renal epithelium of a nephrotoxic poison derived, hypothetically, from the infectious agent of the disease. The histological appearance of the epithelial lesion is also not that of nephrotoxic damage, in which epithelial cells, absorbing the poisonous substance, die and desquamate within a tubule still maintained by an intact basement membrane. There has occurred, rather, a disruption of the entire tubule wall, the tubulorhexic lesion characteristic of the kidney of Acute Renal Failure (10). The inference seems certain, therefore, that the tubular lesions of EHF, both early and late, are the result of circulatory disturbances. This conclusion does not, of course, rule out the primacy of an infectious agent or its products as the cause of the vascular disturbances, for recent experiments by Thal (17) in the production of bilateral cortical necrosis by means of staphylococcus toxin show how this may be produced in the kidney and so result indirectly in extensive tubular damage. There are many similarities between the nature of the tubular lesion in his experiments and what is seen in the kidney of EHF; the differences in topographical distribution seem less important, for, as Thal points out, "cortical necrosis" is not so restricted as the descriptive term would suggest, nor are the tubular lesions of EHF limited to the medulla.

There is evidence that the circulatory disturbance acts on the tubule by means of two deleterious forces; the first, and perhaps less important, is physical pressure, and the second is anoxia following the congestive stasis and the infiltrative intertubular hemorrhage which has produced a cessation of circulation in the involved areas.

Evidences of pressure, especially on the more sensitive proximal convolutions, were seen in the earliest stages of the renal lesion where there was only a simple and moderate congestion of the terminal vessels of the subcortical zone. With an increase in vascular distention the effect of increasing intertubular pressure was evident in the compression and consequent entrapment of proteinaceous fluid in the lumens of the terminal proximals that lay in the involved area (Figure 13 and Plate II), and its final effect is demonstrated by the "intrarenal hydronephrotic" tubular dilatation which ultimately develops in the definitively oliguric kidneys (Figure 24). This dilatation involved all the tubules lying in the cortex above the subcortical zone of pressure and included not only proximal convolutions which are, in regard to the direction of urine flow, proximal to the zone of compression in the medulla but also the distal convolutions, cortical ascending limbs and peripheral collecting tubules which lie distal to it.

This apparent paradox of postobstructional dilatation arises from current misconceptions of the topographical arrangement in the human kidney of nephrons of varying length. In most diagrams (Peter, 18, Figure 63) two types of nephrons are shown, short-looped and long-looped, both of which pass through the outer stripe of the outer zone which in EHF is the zone of hemorrhage. If this were an accurate and complete picture of the human kidney, the distal convolutions and collecting tubules would be cut off from the source of glomerular filtrate and could not dilate. As a matter of fact, the human kidney contains a very considerable number of nephrons similar to those shown in Peter's diagram of the pig's kidney (18) which turn within the cortex and never reach the medulla. An example is given in our Plate VIII, A to C; many have been shown elsewhere (19).[4]

In a kidney with a zone of subcortical obstruction glomerular filtrate can therefore flow through uninterrupted cortical nephrons to the point of origin of the collecting system (*cf.* Plate IXA) and from thence to all distal portions of the neighboring nephrons which are connected with it, even when they have been shut off from their normal source of filtrate by the zone of medullary hemorrhage. This retrograde flow is in fact favored by the obstruction in the lower collecting system which itself passes through the obstructing zone of hemorrhage.

The question may be asked why then do not all kidneys which suffer extensive disruption of their nephrons, such as, for example, Case 28 of the Transition Period, show extensive tubular dilatation in their cortices. The answer would seem to be that for dilatation to occur there must be an adequate source of filtrate; a comparison of Figures 20 and 24 suggests that in the former case the extreme cortical congestion and hemorrhage is incompatible with a sufficient glomerular circulation and consequent formation of filtrate. Thus a lesion which interrupts tubules in the medulla yet spares the circulation in glomeruli is necessary for the development of the tubular pattern observed in the Phase of Established Oliguria.

The discussion of these minutiae concerning fluid flow in damaged nephrons would seem to warrant attention not so much for an importance inherent in their functional effect—for in both the situations described above urinary output was similarly lessened—but as illustrating how the same or similar pathological processes can by variation in their temporal or spatial relations modify or even change the direction of the evolution of the renal lesion. In the examples cited it was a spatial relation; in the ensuing Phase of Diuresis it is temporal. In any case, the simple statement that this lesion or that is present in a section of kidney is meaningless as a description of the structural-functional renal status.

The structural evidence of physical impedance to the flow of urine through the nephrons is of

[4] Work in progress in this laboratory on quantitative aspects of the morphology of nephrons shows that their classical subdivision in the human kidney into two arbitrary groups of "long-looped" and "short-looped" nephrons is a considerable and, as in the present case, at times misleading oversimplification. Our findings show that the loops of nephrons are indeed of varying length but that this variation can be described by a normal frequency distribution curve rather than by absolute categories; in short, some loops are longer than others.

interest to others in addition to the theoretical pathologist. The clinical observers who noted varying degrees of reduction in urinary output in the earlier Hypotensive Phase recognized the distinctive and definitive character of the oliguria which ensued in the later period, and so designated it to the Phase of Established Oliguria. The structural findings show that the distinction is real, since it is based on a difference in causal mechanisms. In the earlier Hypotensive Phase no structural lesion other than evidences of disturbed circulation was present in the kidney to account for decreases in urinary volume, and it is therefore reasonable to suppose that factors such as variations in tissue hydration, cardiac output, renal blood flow and glomerular filtration were responsible for this variable oliguria. In the later phase, what "established" the oliguria is clearly evident in the compression and destruction of tubules and the obstructional dilatation of nephrons that followed.

Of greater importance than pressure effects in the production of tubular damage is the anoxia that results from the stagnation and cessation of blood flow in the widely dilated and abnormally permeable intertubular capillaries of the subcortical zone. It would appear likely that the blood is moving with some difficulty under such circumstances as are shown in an earlier period in Figure 6, where crowded red blood cells are packed to a solid hyaline mass. Any doubts that circulation may cease entirely under these conditions have been answered by the conclusive experimental demonstration of Thal (17), who injected India ink into the general circulation of the living rabbit with an analogous renal "stagnation hyperemia" and found that it did not penetrate into vascular channels which were similarly distended and packed with red blood cells. The further evolution of the congestive lesion in EHF through diapedesis to frank, diffuse intertubular hemorrhage ends, therefore, in stasis.

Under the conditions here described we are dealing with what Ricker, in his classical study of the nature and effects of local circulatory disturbances (20, p. 96 *et seq.*), would have called "rote stasis," and from the late irreversible stage of this rubrostasis he would have derived the "Sequestrationsnekrose" that interrupted the course of the nephrons. It is of particular interest in our problem that in his examination of these effects in various tissues and organs he chose, in the case of the kidney, the necrosis that is produced experimentally by injection of vinylamin. This toxic agent differs from other renal poisons by producing necrosis of tubules in the medulla rather than in the proximal convolutions of the cortex.

Ricker points out that the lesion of vinylamin poisoning begins in the papilla with a "prästatisch" hyperemia followed by diapedesis of red blood cells, and progresses to "Stasis" and eventual "Dauerstasis." The vascular disturbance in his experiments extended to the subcortical zone, and in one instance to the cortex, with resulting necrosis of tubules in the involved areas. His conclusion that the tubular damage cannot be the result of either a functional secretion or absorption of the poison by epithelial cells, as was suggested by Oka (21), or due to specific "affinity" for the poison as Levaditi (22) assumed, has been convincingly confirmed by subsequent evidences that proximal convolutions, Henle's loops and collecting tubules cannot be considered functionally similar; hence it would not seem likely that they should all absorb a poison similarly or, much less, be given to the same "affinities." We can accept, therefore, Ricker's explanation that the tubular necrosis is a phenomenon of ischemic sequestration due to the anoxia of the vascular disturbance;[5] with his broader conclusion that local circulatory disturbances are mediated through the nervous system we need not here be concerned.

THE RENAL LESION IN THE PHASE OF DIURESIS

(Intertubular Hemorrhage; Tubular Disruption with Evidences of Resolution, Regeneration and Repair)

The remaining cases in this series show structural changes that should be considered as illustrative of the effects of the passage of time on the

[5] Ricker also compares the action of vinylamin with that of mercury, which damages the proximal convolutions; this effect he also derives from circulatory disturbances and not from direct nephrotoxic action on the epithelium. Here, modern studies, histochemical and pharmacological, do not support his contentions, though it should be noted that he anticipated a later demonstration (10) that the vascular element of ischemia may play an important if subsidiary role in the production of tubular damage (tubulorhexis) in renal lesions of poisoning. A recent study by Lindegård and Löfgren (23) has confirmed and extended these observations by demonstrating an irregular cortical ischemia not only in the anuric phase of mercuric poisoning but also in its diuretic phase.

fully developed lesions of the Oliguric Phase rather than as representative of the exact picture of the Diuretic Phase observed by the clinician during the recovery of the great majority of his cases. If the individual in the Oliguric Phase does not die of secondary shock, dehydration, electrolyte imbalance or infection by the eighth to tenth days, but survives another week or two, the processes of resolution, regeneration and repair, as in all pathological complexes, begin their automatic operation. A part of these mechanisms of restitution is the functional phenomenon of diuresis. As the pathologist sees them, the progress of these processes, even if in the right direction, is blind and in the end futile, and the individual dies of a renal failure often obscured by some complication such as the infection of a bronchopneumonia, the hyperkalemia of electrolyte and fluid imbalance, or a cerebral accident. It would seem unlikely that these complications can be merely incidental to the renal lesion, but rather it seems that in some manner, either directly or indirectly, renal insufficiency is the basis of their development; for in all the examples studied, though the complications were various, the renal lesion, with its grave structural damage and evidence of functional inadequacy in a BUN elevated at times to over 400 mg. per cent, was constant.

What happens then in the great majority, 95 per cent of all cases of EHF, has not been seen; the description of the typical kidney in the survivor must therefore be deduced and reconstructed on a hypothetical basis, and will be further considered in our discussion.

* * * * *

As happens not infrequently in the descriptions of passage from one phase of a disease to the next, our first case, D, might well have been classified as an example of a renal lesion at the end of the Oliguric Phase; he survived, however, 15 days and as the urinary output, though continuing low, increased somewhat on the last day but one of his life, the description was clinically placed under the title of the Diuretic Phase and will be here so considered. As will be seen, there is no evidence of restitution of disrupted nephrons and in general the renal lesion resembles that observed in the previous phase.

This patient was admitted on the fourth day of his illness with a typical history of the Febrile Phase. The next day he abruptly developed a 4 + proteinuria. From the seventh to the tenth day he was in the Hypotensive Phase with repeated shock that responded poorly to treatment; an established oliguria followed but the urine volume increased from around 15 cc. in 24 hours to 250 cc. on the day before his death. On the twelfth day dialysis by the artificial kidney reduced the serum K from 6.9 to 4.2 mEq. per L. but the patient lapsed into coma, his temperature rose and coughing with bloody purulent sputum developed; a bronchopneumonia was confirmed by X-ray examination. On the day before his death, the fifteenth day of illness, the BUN was 484 mg. per cent.

At autopsy the kidneys weighed 300 gm. and showed the typical and fully developed lesion of subcortical congestive hemorrhage with deep medullary involvement. There was a bilateral bronchopneumonia with multiple small abscesses containing Gram positive cocci, scattered hemorrhages in the right atrium, gastrointestinal tract, the cerebral hemispheres and pons and the anterior pituitary.

Histological examination showed that congestive hemorrhage was extreme in the subcortical zone and had extended irregularly and deeply into the medulla, reaching the papillae (Figure 28). In the outer zone there was an almost complete destruction by necrosis of all tubules contained in the areas of hemorrhage, and these, coalescing, separated cortex from medulla (Figures 29 and 30). Extending downward into the infarct-like area of hemorrhagic infiltration could be seen wisp-like unoriented remnants of straight tubules; their walls were disrupted and in great part bare, but scattered along the denuded basement membranes were huge, atypical nuclei with intensely stained chromatin (Figure 31). Mitotic figures were frequent. The intensity of this excessive regenerative proliferation distorted the tubular configuration so that it was impossible to accurately identify the parts of the nephron concerned in histological section.

In the cortex the characteristic dilatation of the Phase of Established Oliguria was still present (Figure 32), involving Bowman's space and both proximal and distal convolutions; it will be recalled that in this patient's "diuresis" only 250

cc. per 24 hours was draining from the renal tubules. The epithelium of the proximal convolutions was not only thin but cellularly atypical; its nuclei were not round, but oval, and were irregularly distributed in the tubule wall, in some places forming islands of excessive proliferation (Figure 33); the appearance was that of a regenerated epithelium. Moreover, the tubules were more widely spaced than normal (*cf.* Figure 1) and the interstitial tissue was infiltrated with mononuclear cells and fibroblasts. The glomerular tufts were large with widely dilated capillaries that contained discrete, apparently circulating red blood cells.

The topographical aspect of the lesions just described is shown in the dissected nephrons. Plate VIII, A to C, shows a complete cortical nephron with a short loop that did not extend into the subcortical zone of intertubular hemorrhage and is therefore uninterrupted. As in all examples of acute renal failure associated with traumatic and toxic injury, the tubule is damaged from glomerulus to collecting tubule. As is usual, the damage is more severe in the proximal convolution than in the so-called "lower nephron," consisting of scattered areas of tubulorhexic disruption with the lumen containing desquamated epithelial cells. Where disruption has not occurred, the wall is composed of a heavily stained, irregularly thin layer of atypical, regenerated epithelial cells quite different from the plump, evenly contoured cells of the normal tubule (*cf.* Plate I). The transition to the loop of Henle is abrupt and there is, as in all cortical nephrons, only a suggestion of a thin portion. From here on through the loop and ascending limb and distal convolution the tubule is greatly dilated with clear tubule fluid and contains desquamated cells and debris. As is so often the case, the short connecting tubule is less distended.

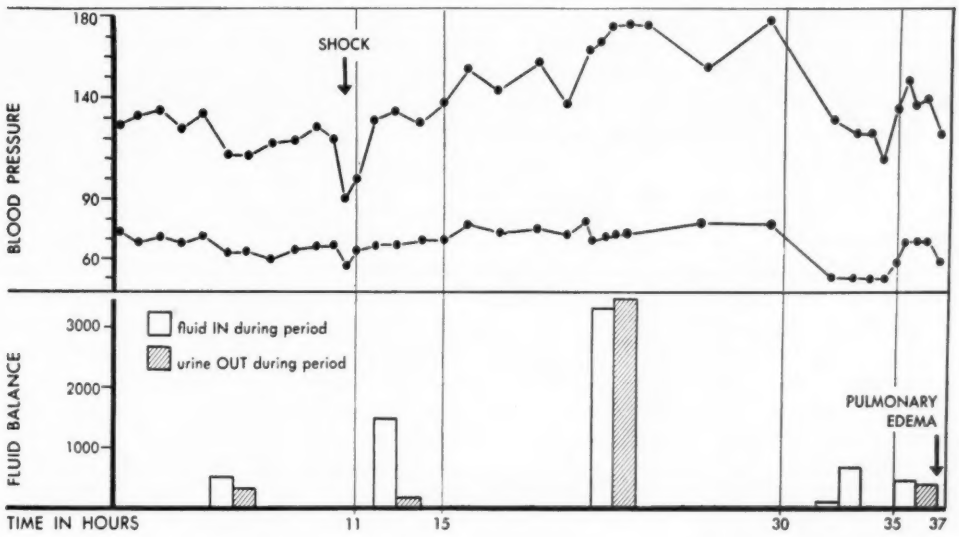
Plate IX, A to E, shows the peripheral collecting system from its origin in a junction of connecting tubules of five nephrons that lay beneath the surface of the kidney to its destruction in the hemorrhagic zone of the subcortical medulla. The connecting tubules of 9 nephrons in all, filled with black stained material that obscures their epithelial pattern, join to form a collecting tubule which is empty. In many instances the collecting tubules contained similar material, but this exceptional example was chosen for illustration because it shows the cellular pattern more clearly. It will be observed that the epithelium is not disturbed until the proximity of the subcortical zone of hemorrhage is approached; scattered particles of cellular debris then appear in increasing numbers until, in the substance of the hemorrhagic zone, a complete necrosis and coagulation of the entire tubular wall is evident. The tuning-fork juncture with a neighboring collecting tubule which is similarly affected is well shown; below this point the single common duct contains black-stained material. The appearance on histological section in other cases of similar, completely necrotic collecting tubules which have nevertheless maintained their external configuration is shown in Figures 22 and 29. The general architectural pattern of the kidney is shown in Text Figure 8 in a reconstruction of the renal lobule composed of camera lucida tracings of dissected nephrons.

In Case 43 a frank diuresis had been established for the last five days of the patient's life, and the renal lesion has special interest because, of all the examples of this phase of the disease that came to autopsy, in this one alone resolution of whatever structural damage had occurred had reached at least the point at which one might imagine a chance of eventual functional restitution to be possible. The resulting "kidney" of such a problematical restitution would, however, have been severely deficient in its "reserve" of nephrons.

The patient entered hospital on the third day of his illness in the Febrile Phase with a temperature of 103.6°, and on the fifth day went into typical shock of the Hypotensive Phase with the hematocrit reaching 66 per cent. With appropriate treatment he passed through this episode to the Oliguric Phase, in which his average daily urinary output was approximately 100 cc. with a BUN rising to 280 mg. per cent. His blood pressure also rose (170/106 mm. Hg), and the hypervolemic syndrome accompanied by 5 convulsions followed. Daily urinary output increased rather abruptly in 24 hours on the twelfth day from 190 to 1000 cc. and reached 4600 cc. the day before his death. During this terminal period he presented the difficult problem in water and electrolyte balance of a "limited homeostasis" illustrated in Text Figure 9, so that the margin between dehydration with the threat of secondary shock and ample hydration with impending pulmonary edema was represented

TEXT FIG. 8. A RENAL LOBULE FROM CASE D WHO DIED ON THE FIRST DAY OF THE DIURETIC PHASE

Note the persisting subcortical hemorrhage with destruction of tubules. Heavy lined proximals indicate regenerated atypical epithelium (*cf.* Figure 33 and Plate VIII A) ; other symbols as in Text Figure 5. Magnification 18×.



SUMMARY OF FLUID BALANCE FOR 37 HOURS

IN:	TOTAL FLUID	+ 5660 ml
OUT:	Urine	− 4930 ml
	Vomitus	− 570 ml
	TOTAL	− 5500 ml
GROSS BALANCE		+ 160 ml
INSENSIBLE LOSS		− 750 ml
NET BALANCE		− 590 ml

TEXT FIG. 9. CASE 43—EXAMPLE OF LIMITED HOMEOSTASIS DURING DIURETIC PHASE OF HEMORRHAGIC FEVER

These observations were made on fourteenth and fifteenth days of illness. Death occurred on the sixteenth day, 24 hours after study. The patient was very dehydrated at the start of the observations, having been in negative fluid balance for many days. Note the response of shock and blood pressure to increased fluids between the eleventh and fifteenth hours. Also note decrease in blood pressure between the thirtieth and thirty-fifth hours when output was allowed to exceed intake. Pulmonary edema occurred in last period when intravenous fluids were increased even though net balance was just barely positive during this time. (Note: Right-hand column, bottom line in 30 to 35-hour box should be cross-hatched.)

by a net negative fluid balance of 600 cc. During episodes of hypotension and shock the diuresis disappeared and urinary output was at oliguric levels, to rise again to 4000 cc. when arterial pressure was restored by nor-adrenalin. The last BUN taken the day before his death had decreased to 173 mg. per cent, and there was only a trace of protein in a urine of 1.011 specific gravity.

At post mortem there was found a confluent bronchopneumonia, hemorrhages in the right atrium, cerebral hemispheres and basal ganglia, and in the anterior pituitary. The gross appearance of the renal lesion is summarized in the autopsy protocol as "lower nephron nephrosis, slight."

Histological examination showed a marked contrast to the pattern of a general dilatation of all cortical tubules seen in the previous stage of established oliguria; the lumens of the proximal convolutions and other cortical tubules were not widely dilated but contained some granular material, as did Bowman's spaces of glomeruli which were otherwise normal (Figure 34). The proximal convolutions were lined with an epithelium of the original normal type; there was little evidence of any regenerative renewal of cells, such as the irregularity in thickness and nuclear size and shape and staining affinity that characterized the epithelium of proximal convolutions in most cases at this stage of the disease. The general appear-

ance was therefore of a cortex which had never been greatly damaged.

Contrasting, was the alteration in the subcortical outer stripe of medulla. Extending downward from the cortex into this zone, which had the appearance of a region of resolution of hemorrhage and replacement fibrosis, were the thickened terminal segments of proximal convolutions; their epithelium was quite atypical, varied in thickness and containing many irregularly distributed large nuclei (Figure 35). In the broad band of the affected region irregular foci of typical hemorrhagic infiltration persisted, alternating and blending with ill-defined areas where the tubules were separated not by masses of red blood cells but by cellular fibrous tissue (Figure 36); a considerable increase in collagenous fibrils surrounded atrophic tubules and fused with their thickened basement membranes (Figure 37). Extension of the fibrosis could be traced upwards into the lower cortex in the form of cellular scars which replaced tubules and surrounded glomeruli (Figure 38).

The renal lesions in five cases—two of which, 16 and 46, died on the fourth day of diuresis, two others, 11 and 19, on the ninth day, and one, 29, on the eleventh day—showed essentially similar lesions to those just described but with more evidence of previous damage and a lesser degree of restitution. In the subcortical zone of the medulla, intertubular hemorrhage still persisted in all instances with intermingled areas of resolution and interstitial reaction (Figure 39). In two of the five cases, 16 and 46, the epithelium of the cortical proximal convolutions was of the original mature type; in three, 11, 19 and 29, it resembled a replacement by atypical regenerated epithelium similar to that shown in Figure 40; in none were there the marked distention and dilatation of tubule lumens observed in the preceding Phase of Established Oliguria.

The last two cases were of the longest duration that occurred in this series and therefore present the maximum effect of the passage of time on the renal lesion that was available for observation.

The first of these, Case 33, died on the nineteenth day of his disease in the tenth day of the Diuretic Phase. He had passed through the Hypotensive Phase with two episodes of shock which responded to appropriate therapy, and an Oliguric Phase of 4 days in which his urinary output averaged 250 cc. in 24 hours when the BUN reached a maximum of 286 mg. per cent. During this period he was hypertensive (158/112 mm. Hg). On the ninth day of his illness, diuresis began abruptly and continued until his death on the nineteenth day, the urinary output ranging from 1410 to 4400 cc. in 24 hours. The last days of his life were an example of homeostatic instability with repeated episodes of dehydration, hypotensive shock, fluid replacement and threatening pulmonary edema. During this terminal period he received 11 units of serum albumin. Cerebral involvement became apparent, his temperature reached 104° and he died on the nineteenth day of his illness. The BUN on the day preceding death had decreased to 145 mg. per cent.

At autopsy a confluent bronchopneumonia in addition to hemorrhages in the right auricle, pituitary, and interventricular septum were present. The renal lesion is described as showing "a fairly marked degree of congestion and focal hemorrhage in the renal medulla."

In the histological sections the proximal convolutions in the cortex were lined with an atypical epithelium. The lumens were therefore irregularly widened and contained a moderate amount of coagulated material and desquamated cells (Figure 40). The contrast in appearance between these tubules whose lumens were somewhat increased by the irregularity of their epithelium and the frank and even distention of a tubule dilated by internal pressure, as occurred in the Oliguric Phase, may be seen in a comparison of Figures 40 and 24, and of Plates X and VII. Bowman's space was not dilated and the glomerular tufts appeared normal. The tubules of the cortex were widely spaced and the interstices filled with a loose fibrous connective tissue in which were clusters of round cell infiltration. In the subcortical zone of the medulla and extending to the papillae extensive areas of persisting intertubular hemorrhage were seen; in these areas the smaller tubules were widely separated and apparently reduced in number, but the larger collecting tubules were not only intact but showed an extreme and irregular proliferation of their distinctive epithelium, which formed irregular plaque-like formations distorting the normally even contours

of their lumens (Figure 41). The resulting distortions of the terminal collecting tubules and the ducts of Bellini entering the pelvis, the latter filled in part with renal failure casts, are shown in Plate XI.

Case 18 survived 27 days; he had passed through the typical Febrile and Hypotensive Phases of the disease with only moderate shock. On the seventh day of his illness he entered a 6-day period of Established Oliguria with hypertension and a rising BUN that continued without remission until his death, reaching a final figure of 329 mg. per cent. On the twelfth day his urinary output increased from 195 to 2175 cc. in 24 hours and a moderate but irregular diuresis, at times of 3000 cc., continued to his death. During this terminal period the usual problem of maintenance of fluid balance between dehydration and pulmonary edema was present with repeated episodes of secondary shock. In this period he received no serum albumin.

At autopsy, a marked hypostatic congestion and edema of the lungs were present with the usual hemorrhages in right auricle and pituitary. The renal lesion is described as showing the "classical medullary hemorrhage with some evidence of resorption."

Histological examination showed extensive intertubular hemorrhage in the subcortical region extending deep into the medulla. The areas of hemorrhage were not continuous, but formed an irregular zone in which regions of tubular collapse and dilatation alternated with definite fibrosis of the intertubular tissue. The dilated tubules lying in the fibrous areas extended by medullary rays into the cortex and were either empty or filled with hyaline material. Proximal convolutions in the cortex were irregularly dilated; their epithelium was of a normal mature type. The glomeruli, save for coagulated material in Bowman's space, were not remarkable.

* * * * *

All of the individuals surviving the phase of oliguria for periods varying from 1 to 16 days showed widespread evidence of the usual renal reparative processes of epithelial regeneration and replacement fibrosis; the general dilatation of cortical tubules was not present. The structural repair concerned the kidney tissue rather than the nephrons, for there had occurred no restitution of organ structure in those elements whose continuity had been destroyed. In Case 43 the histological pattern of the cortex with its glomeruli and proximal convolutions appeared not greatly abnormal (Figure 34) and, at least from the structural viewpoint, had the appearance of a possible functional adequacy even though renal insufficiency was still evident in the BUN which had decreased from a maximum of 280 to 173 mg. per cent. Judging from the amount of scattered scar tissue and distorted tubules in both the subcortical zone and in the cortex, a considerable number of nephrons must have been in part destroyed.

* * * * *

Further evidence of the nature of the repair is found in an individual who, having recovered from EHF, died of causes other than renal insufficiency 149 days later.

This patient was first admitted to hospital on the third day of his illness with the typical clinical picture of EHF. On the next day, with a blood pressure of 110/90 mm. Hg, a pulse of 112 and a hematocrit of 60 per cent, he was given one unit of serum albumin; from then on the blood pressure was stable. On the sixth day, the urinary output dropped to 25 cc.; on the seventh it was 125 cc.; he then became hypertensive (150/100; 170/100 mm. Hg). On the tenth day diuresis of 1700 cc. ensued; the BUN, which had risen to 70 mg. per cent, fell to 43 on the twelfth day. He was transferred to a hospital in the States where, 79 days after the onset of his illness, a test of his maximum concentration capacity showed a specific gravity of 1.010. During this stay in hospital the patient presented symptoms of adrenal insufficiency, cardiac irregularity and occasional "clonic seizures." In one of these he died, 149 days after the onset of the attack of EHF.

At autopsy the anterior pituitary was found to be almost entirely destroyed by an old hemorrhagic infarction; the adrenals weighed 6.2 gm.; in histological section there was a thinning of the cortex with a marked lipoid depletion and areas of necrosis in the zona glomerulosa; the medulla was well preserved. The other endocrine glands, save for atrophy and maturation arrest of spermatogenesis in the testicles, were not remarkable.

The kidneys weighed 370 gm.; the surface of one was described as smooth, the other as finely granular.

Histological examination showed similar lesions in both kidneys, but the cellular scars to be described were more prominent in one. The subcortical zone of the medulla, which from the history may be assumed to have been the seat of circulatory disturbances, showed an increase in intertubular fibrous tissue and a consequent wide spacing of the tubules passing through it. The majority of these straight tubules appeared normal, but scattered through the denser areas of fibrosis were collecting tubules which showed a hyperplastic proliferation of their epithelium (Figure 42). The deeper parts of the medulla, including the papillae, were free of fibrosis and appeared normal. Rather different from the acellular fibrous scarring were other more recently appearing wedge-shaped areas of monocytic infiltration and fibrous proliferation which extended from mid-medulla through the cortex to the surface of the kidney. Within the confines of these scars remnants of atrophic tubules and glomeruli were visible (Figure 43).

* * * * *

In the kidneys of this individual who had recovered from the acute episode of EHF there are two types of fibrous scarring and tubular destruction. The one, present in the subcortical zone, is composed of an acellular fibrosis; the other, extending from medulla to cortex, is relatively afibrous and is filled with an infiltration of inflammatory monocytic cells. The former corresponds in location and in its fibrous components with that seen in the reparative process in cases of shorter duration. The frankly inflammatory nature of the latter type of fibrosis seems to be either a new element in the pathological complex or a greatly exaggerated one, since in the cases formerly described cellular infiltration of an inflammatory reaction, though occasionally present, was at a minimum. In the present case the noncommittal diagnosis of sub-acute pyelonephritis is at least warranted; it is a matter of speculation what relation it bears to the original lesion of EHF which appears to have healed with a benign fibrosis and the destruction of a certain number of nephrons.

RÉSUMÉ

Concurrent with our description of the structural changes in the kidneys during the succeeding phases of EHF have run a discussion of their significance as factors in the renal status and a critical consideration of the pertinent literature. What now follows is an uninterrupted recapitulation of the course of the disease, attempting a chronological synthesis in structural-functional correlations that will integrate the renal lesion into the clinical syndrome.

During the first hours of the Febrile Phase the individual shows clinical evidence in the intense flush of the skin and visible mucous membranes of vasodilatation of peripheral small vessels. The capillaries of the nail folds are widely dilated; the same is apparently true of the small vessels in the renal vascular bed, as ERBF is either normal or increased. It can be assumed, therefore, that the kidney during this first stage is flooded with a rapid circulation of blood through vessels which are, save for vasodilatation, essentially normal. Since only traces of protein are found in the urine, it would seem that the glomerular capillaries have not as yet suffered the characteristic and catastrophic lesion of the disease which is to develop in the next stage.

This vascular disturbance is revealed in the succeeding Hypotensive Phase by the abrupt leakage of plasma from the capillaries, and results in a reduced circulating blood volume. It is associated with decrease in arteriolar tone so that hypotension and shock are the result. The structural-functional correlation at this point is clear, for at autopsy the escaped plasma is found in the retroperitoneal spaces.

Lowered arterial pressure of shock is a sufficient cause of decreased renal blood flow, but there are also local disturbances in the renal vascular system. Proteinuria abruptly increases, often in the course of a few hours, as evidence of leakage of plasma through glomerular capillaries that have undergone damage similar to that which is so widely spread throughout the tissues; the correlative structural aspect of the lesion is seen in the precipitate which fills Bowman's space and in the areas of intertubular edema, analogous to the retroperitoneal edema, that are found in medulla and cortex. Clearances show a sharp reduction in

ERBF even in the absence of clinical shock; if the latter concomitantly or subsequently occurs, as it did in most of the fatal cases, a summation of general and local effects operates to reduce renal blood flow.

It is the failure of renal blood flow, previously either abundant or excessive in an engorged vascular bed the arterioles of which have lost their tone and whose capillaries and venules are permeable to plasma but not to red blood cells, that is responsible for the origin of the characteristic renal lesion of the disease. The distinctive localization of this circulatory disturbance is due to the anatomical and functional peculiarities of the vascular system of the kidney which have been previously described. Even with a reduced blood flow, the atonic and permeable vessels of the "medullary sponge" in the subcortical zone of the medulla, a region shown to be the last to suffer ischemia, are flooded and distended to the point of tubular compression. At this stage the circulation in cortex and deep medulla is not greatly disturbed.

The status of the renal circulation is now that which Ricker (20) would have called a "prestatic" congestion, for, judging from histological appearances, the blood within the intact dilated intertubular capillaries is circulating. There is little structural or functional effect other than moderate pressure on the tubules in the region of subcortical congestion. Urinary output has been irregularly and variably reduced and follows, in the main, alterations of blood pressure and consequent renal blood flow. In clinical terms, oliguria has not been "established."

In the Transition Period and Oliguric Phase, clinical evidences accumulate of an intensification of the general damage to small vessels and the consequent escape of more than plasma. Petechiae in the skin increase to a maximum on the fifth or sixth day; ecchymoses or hematomata at the site of trivial trauma, hematemesis, melena and hemoptysis all appear toward the end of the Hypotensive Phase along with a reduction in platelets. So in the subcortical zone of the medulla, hemorrhage, at first by diapedesis and in the end by capillary disruption, infiltrates the renal parenchyma. All tubules in the areas of involvement now lie widely separated in a mass of non-circulating red blood cells.

The circulatory disturbance has now passed from Ricker's prestatic phase to that of rubrostasis and is accompanied by necrosis of those portions of the nephrons which lie in the anoxic areas of congestive stasis. This interruption of the intrarenal passages which tends to isolate cortex from medulla is not complete, however, for it is formed by the coalescence of focal areas of congestive hemorrhage which arose from the peculiar horsetail configuration of the *arteriae rectae* of the lower glomerular efferents. The tubules of many nephrons and of the collecting system may be preserved even in what appears in section to be a severely affected kidney. The importance of intact channels through the area of destruction for the continuation of some flow of urine in the Oliguric Phase and for its increase in the Diuretic Phase which may follow, is apparent.

The clinical observation of the development of an established oliguria during this period correlates with the pathological finding of compressed and interrupted intrarenal channels. Not only are the compression and disruption of tubules seen in the subcortical zone of intertubular hemorrhage, but, more conclusive, the effect of that obstruction is evident in the consequent dilatation of cortical tubules, both proximal and distal, that lie above it. Such retrograde alterations in the course of urine flow through the kidney are made possible by the presence of intracortical nephrons which have escaped medullary disruption. It is noteworthy that this distention disappears with the onset of diuresis, for it is not present in the kidney of those who have passed through a similar period and died in the Diuretic Phase.

Tubular destruction is not limited to the subcortical zone of congestion; stasis in the *arteriae rectae* reaches the papillae and the tissue lesions of hemorrhage and necrosis follow. Extension into the cortex is less obvious, yet nephrons from severely damaged kidneys regularly showed the tubulorhexic, disruptive lesions of anoxia throughout the proximal convolutions.

Those who died in the Phase of Established Oliguria and in the following Phase of Diuresis showed such extensive damage to both nephrons and collecting system that any adequate structural restitution in their kidneys would seem to have been impossible. We have previously considered in detail the mechanisms of both struc-

tural and functional recovery after the tubular necrosis of acute renal failure (24); it is unlikely, therefore, considering the broad extent of tubule that may be destroyed by the tubulorhexic lesion, that the repair of the damaged nephrons in EHF is possible. Healing, as has been demonstrated in the case of acute renal necrosis, is due to formation of scars that contain afunctional remnants of destroyed nephrons.

Survival of the individual and ultimate recovery therefore depends on the escape of an adequate number of nephrons from anoxic necrosis. Since 95 per cent of the individuals with EHF survive and, in routine clinical examination during convalescence, appear to have recovered normal renal function, it follows that the great majority never could have developed the extensive structural damage that was present in those who died in oliguria or later diuresis. In the typical case of EHF the turning point towards recovery or exitus comes in the Transition Period; if the vascular disturbance remains at the level of a prestatic congestion with little or only moderate intertubular hemorrhage and consequent destruction of a few nephrons, then a return of adequate blood flow is the major requisite for the restitution of the renal status. These relations are illustrated graphically in the lowest subdivision of Text Figure 1. Individuals recover from the renal lesion of EHF not by repair of nephrons but because a great number of their nephrons have not been irreparably damaged.

How frequently future difficulties in renal function are to be anticipated in those who have survived an attack of EHF remains uncertain until exact measurements by clearances of suitable cases have been accumulated. In all but the milder cases some nephrons have most likely been destroyed, and it is known that after most forms of acute renal failure, in spite of some hypertrophy and hyperplasia of the survivors (24), this loss is demonstrable for a considerable time (25). Whether a kidney with such a lessened "reserve" is a potential hazard remains speculative; the one case available for examination in this series showed the lesions of a sub-acute pyelonephritis.

Since the Transition Period and the Oliguric Phase is the time of renal crisis, it would be most helpful if some distinguishing clinical characteristic or laboratory procedure might be discovered that would differentiate the passing "renal insufficiency" occurring in the Oliguric Phase in the individual who ultimately is to recover, from the definitive "renal failure" of one who has an irreparable destruction of nephrons and is to die. Can the former temporary insufficiency be considered, for example, a "functional" phenomenon in some nature different from an "organic," irreversible failure, and if so cannot the two sorts of disturbance be distinguished by a refinement of clinical or laboratory technique?

These questions of differential diagnosis which, though they certainly have metaphysical implications that need not here concern us, would, if answerable, be eminently practical. The problem has been examined under experimental conditions in the perfused frog's kidney, where a simplification of the factors involved, if extreme, at least makes its consideration possible (26). If the conclusions of these experiments are accepted, that which has been assumed to be two different sorts of disturbed renal activity, "functional" and "organic," are not similar; they are in fact one and identical and no elaboration of technique can make them two. It is true that because of present limitations in the scope of morphological techniques the visible structural aspect of the situation at times differs; if one can *see* a structural alteration, then the differentiation between the evanescent and the irreversible renal disturbance may be possible. For these reasons it is understandable how an "established oliguria" in the clinical sense was observed in individuals who recovered; in their kidneys some nephrons may have undergone the irreversible structural lesions that characterized the phenomenon in the pathological sense, but not, as in the fatal case, a significant number to preclude recovery of an adequate renal status. To distinguish between the two situations the structural alterations evident in a renal biopsy, which in the present case was impractical, would be required.

In general, the mechanisms of the two variations in urinary output, oliguria and diuresis, are similar in EHF to those which operate in the classical example of Acute Renal Failure associated with various forms of traumatic or toxic injury. In both, a decrease and subsequent restoration of renal blood flow would seem to be im-

mediate factors in the production of decreased and augmented urine flow. As accessory mechanisms that reduce tubule flow in both forms of renal damage, increased intra-renal tension from interstitial edema or swelling of osmotic origin of the epithelium of the proximal convolutions must be considered, and any "casts" that may be present must act as deterrents of tubular flow.

In contrast to these similarities between the nature and causes of decreased urinary output in Acute Renal Failure and EHF one difference has been previously noted; the *establishment* of the oliguria in the latter. Though the factors which establish the oliguria, tubular disruption, are present in all forms of ischemic renal damage, the concentration of its obstructive effect by a zone of subcortical hemorrhage is present only in EHF.

The sudden onset of diuresis, at times from no urine excretion to over several liters in 24 hours, makes it certain that a circulatory phenomenon is concerned and not the restitution of some structural element.

It should be noted that the diuresis observed in the fatal cases was not the flood that occurred in those who recovered. In the latter instances the urinary output commonly stabilized at 6 to 8 liters per day with an exceptional output of 18 liters. In the fatal cases here reported, only an occasional individual passed as much as 4 liters and the more common daily output was around 2. This depression in fatal cases of a diuresis that was "normal" in the recovering case is explicable by the predominating and persisting effect of the structural alterations making for decreased flow in the Phase of Established Oliguria, namely, intertubular hemorrhage and tubular disruption. Here again is evidence that the cases which recovered did not have the grave disruptive lesion in the nephrons that was observed in the fatal cases.

Variations in urinary volume in Acute Renal Failure and EHF are both "glomerular" and "tubular" in their origins. The glomerular functional mechanism is relatively simple, the tuft filtering more or less in accord with the flow and pressure of blood circulating through its capillaries. Its functional response to the return of circulation is immediate, since its structure has not been greatly altered and filtration is a simple process. Return of tubular function, absorption of electrolyte and water, is slow because of the need of elaborate reconstitutions of cellular mechanisms, such as the mitochondrial apparatus (24). Hence it is the temporal relation of varying responses, prompt filtration and delayed reabsorption, to one event, the return of circulation, that determines the final effect, *i.e.*, diuresis and loss of elements which are normally conserved.

The tubular element in abnormal variation of urine output is more complex, even if we ignore the obligatory phase of water absorption which is presumed to occur in the distal portions of the nephrons or collecting ducts. To do so in our problem would seem permissible, since the magnitude of output during the Diuretic Phase points to trouble in that portion of the nephron where we know by direct observation (27) that 80 per cent of the glomerular filtrate is absorbed, namely, the proximal convolution.

The anomalous and paradoxical effects of disturbances in tubular function are apparent enough when attempts are made to examine them indirectly by means of "clearances" done in mammalian experiments; it is perhaps equally optimistic to pass to the other extreme of the situation, in which the experimental oversimplification of examining the problem in the perfused frog's kidneys may seem excessive. However, certain phenomena become apparent under these conditions which are at least suggestive in a hypothetical consideration of the problem in man.

If a frog's kidney, lying *in situ*, is perfused by the renal artery and renal-portal vein with modified Locke's solution containing glucose, a normal "urine" is formed in which the effects of tubular function are evident in the hyposthenuria, the absence of sugar and, if present in the perfusate, the secretion and concentration in the urine of a dye, neutral red. If a poison, urethane or $HgCl_2$, is administered in low dosage to the tubules alone and their functions are thus moderately depressed, there develops a marked increase in volume output, a fall in rate of dye excretion, an increase in total electrolyte elimination, and the appearance of sugar in the urine. If the dosage of poison is increased the urine volume decreases, with no return of sugar absorption or dye secretion, ultimately reaching zero, and the kidney is seen to be swollen and edematous. Histological examination of such perfused kidneys shows no

frank epithelial structural lesions in the "stage of diuresis," and marked epithelial destruction and intertubular edema in the later "stage of oliguria" (28, 29).

In a tentative manner one might imagine analogous tubular disturbances operating in the complexity of the renal lesions of Acute Renal Failure and EHF. Under clinical conditions the sequence of events in the two diseases is reversed, oliguria and structural damage of the grave renal insult preceding a phase of restitution of renal function that is indicated by diuresis when tubules, still inadequate, fail to absorb the fluid of an increasing glomerular filtration that has followed a return of renal circulation.

As this discussion has progressed comparisons and analogies have continuously presented themselves relating the structural and functional aspects of the renal lesion of EHF to those of the renal lesion of Acute Renal Failure which is associated with all the various forms of traumatic and toxic injury. In another place (30) these renal lesions have been compared, in their merging patterns that lie between two types of structural alteration, nephrotoxic and tubulorhexic, to the gradation of spectral bands that, individual, yet blend to a continuum; in this spectrum each case presents its characteristic signature which is derived from the peculiar qualities of its clinical origin. On such a spectrum the distinctive elements of the renal lesion in EHF, such as its prominent Transition Period and the Established Oliguria Phase with its causal relation to the interruption of urinary channels by the characteristic subcortical zone of congestive hemorrhage, stand out in bright lined contrast; appearances both functional and structural, are very similar and yet sharply different from those of Acute Renal Failure. It would seem possible to resolve this apparent anomaly and rationalize our metaphor by the recognition that the renal lesion in EHF is Acute Renal Failure in an individual whose peripheral vascular bed, including the renal, is atonic and permeable as a result of an infectious disease. Thal's experiments (17) have removed all doubts that infectious noxa can produce the vascular disturbance of renal ischemia and its distinctive tubulorhexic necrosis.

There still remains the task of establishing what the exact nature of the infectious noxa in EHF may be. It has been shown that it does not have the characteristics of a histamine-like substance, and preliminary evidences of a specific vasodilator substance that circulates in the plasma have so far proved inconclusive (31). As in the more fundamental problem of the nature of the causative agent in the disease, it would seem that here lies hidden what might prove to be the definitive contribution to the understanding of the renal lesion in Epidemic Hemorrhagic Fever.

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FIGURE 1

FIG. 1. CASE 38, DIED ON FOURTH DAY OF PRIMARY SHOCK AT THE BEGINNING OF THE HYPOTENSIVE PHASE—THE OUTER CORTEX

Except for some swelling of the epithelial cells of proximal convolutions and dilatation of distal tubules, which appear empty, the renal elements are essentially normal. Magnification 150 ×.

FIGURE 2

FIG. 2. JUNCTION OF LOWER CORTEX AND MEDULLA SHOWING DILATATION OF PROXIMAL CONVOLUTIONS ENTERING THE MEDULLA AND OF ASCENDING LIMBS PASSING TO THE CORTEX. MAGNIFICATION 150 ×

FIGURE 3

FIG. 3. MID PORTION OF OUTER STRIPE OF OUTER ZONE OF THE MEDULLA

In the central lower half can be seen the irregular and diffuse pattern of dilated intertubular capillaries crowded with red blood cells. Magnification 150 ×.

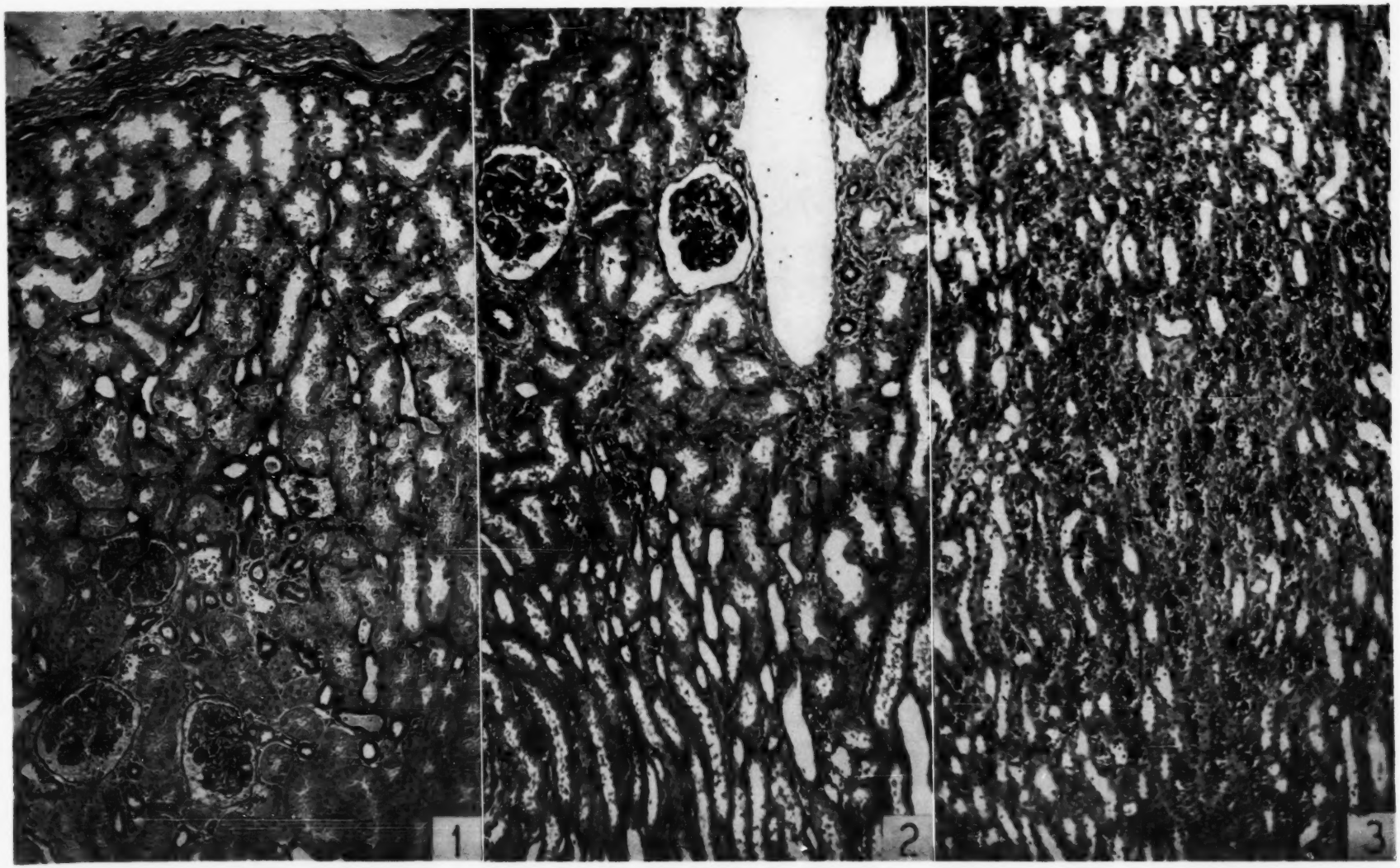
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FIGURE 4

FIG. 4. THE CONGESTED AREA OF THE SAME KIDNEY AS SHOWN IN FIGURE 3

The thin endothelial walls of the intertubular capillaries are intact and their greatly dilated lumens are filled with discrete red cells, which gives the appearance of circulating blood. There is no hemorrhage. Magnification 300 ×.

FIGURE 5

FIG. 5. THE DEEP MEDULLA

The collecting tubules are well preserved; there is no great congestion but there is extensive intertubular edema. Magnification 150 ×.

FIGURE 6

FIG. 6. CASE 9, DIED ON THE FIFTH DAY IN THE HYPOTENSIVE PHASE OF PRIMARY SHOCK—AN AREA OF SUBCORTICAL CONGESTION SIMILAR IN LOCATION TO FIGURE 4

The thin endothelial walls of the intertubular capillaries are intact, their distended lumens crowded with red cells which have fused to hyaline masses, thus giving the appearance of stagnation of flow or stasis. Other cross sections are of tubules with flattened epithelium (thin loops?) containing hyaline casts. Magnification 300 ×.

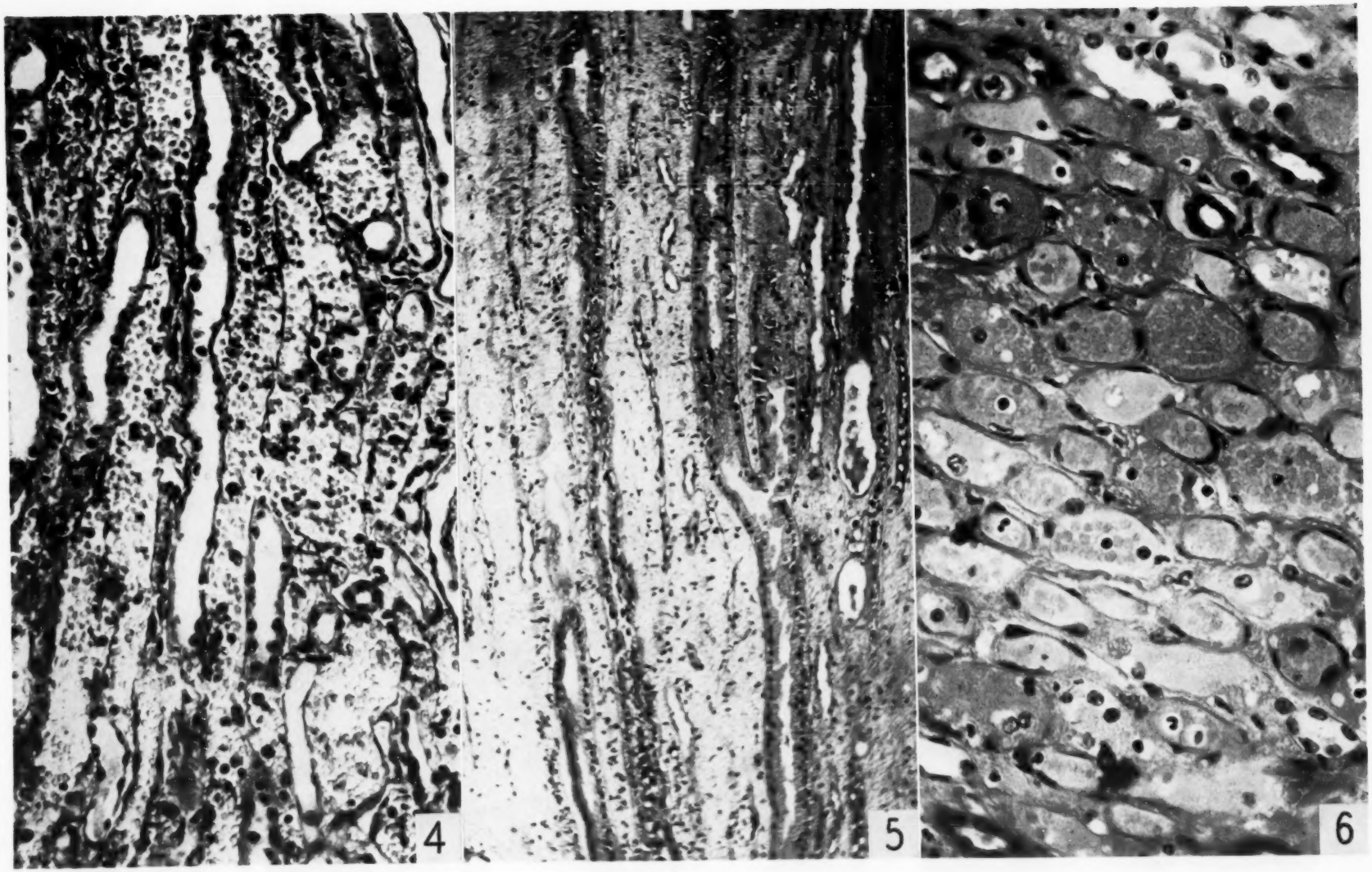
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FIGURE 7

FIG. 7. MID-CORTICAL REGION SHOWING A GREATLY CONGESTED GLOMERULAR TUFT

There is considerable leakage of protein into Bowman's space. In the two cross sections of proximal convolution, beginning swelling and vacuolization are apparent. Magnification 500 ×.

FIGURE 8

FIG. 8. CASE 20, DIED OF PRIMARY SHOCK ON THE SEVENTH DAY IN THE HYPOTENSIVE PHASE—AN AREA OF CORTICAL INTERTUBULAR EDEMA SURROUNDING DISTORTED CROSS SECTIONS OF PROXIMAL CONVOLUTIONS THE EPITHELIUM OF WHICH SHOWS SCATTERED CLEAR VACUOLES—MAGNIFICATION 500 ×

FIGURE 9

FIG. 9. CASE 26, DIED ON THE SIXTH DAY OF PRIMARY SHOCK IN THE HYPOTENSIVE PHASE —EXTREME SWELLING OF THE PROXIMAL CONVOLUTIONS THROUGHOUT THE CORTEX WITH MARKED VACUOLIZATION OF THE EPITHELIAL CELLS—MAGNIFICATION 500 ×

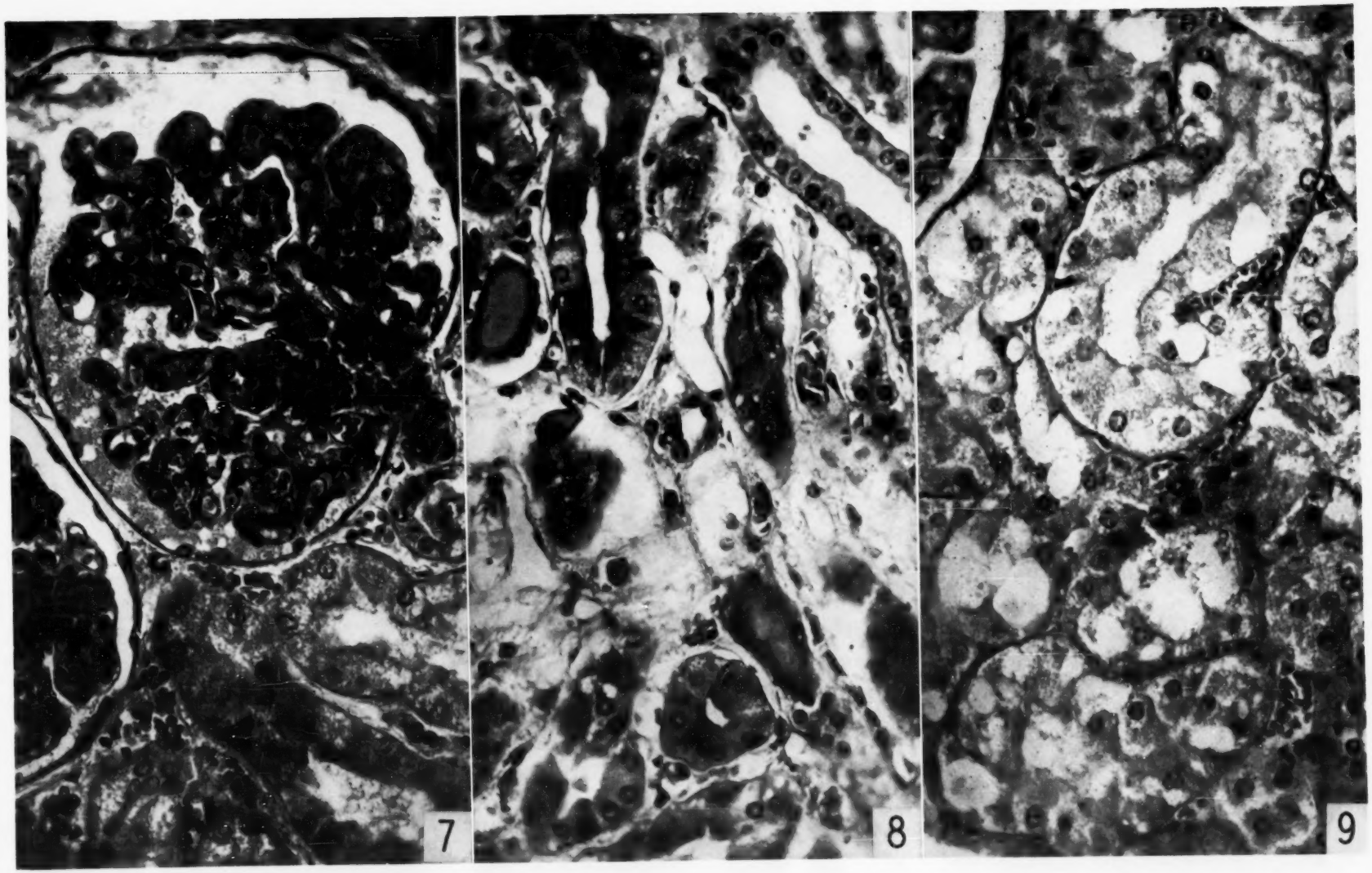
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FIGURE 10

FIG. 10. CASE C, DIED ON THE EIGHTH DAY WITH PULMONARY EDEMA IN THE HYPOTENSIVE PHASE—TRANSITION OF CORTEX TO MEDULLA SHOWING EXTREME FINE VACUOLIZATION OF PROTOPLASM OF CELLS OF TERMINAL SEGMENT OF A PROXIMAL CONVOLUTION

Note the well preserved brush borders. Magnification 500 ×.

FIGURE 11

FIG. 11. VACUOLAR SWELLING OF TUBULAR EPITHELIUM IN CORTICAL PROXIMAL CONVOLUTION WITH EXTENSION INTO BOWMAN'S SPACE AND COMPRESSION OF GLOMERULAR TUFT—MAGNIFICATION 500 ×

FIGURE 12

FIG. 12. CASE A, DIED ON THE FIFTH DAY OF PRIMARY SHOCK IN THE HYPOTENSIVE PHASE—A MEDULLARY RAY IS SHOWN PASSING HORIZONTALLY THROUGH THE MIDDLE HALF OF THE FIGURE

The majority of the tubules (ascending limbs of Henle's loop) are filled with clear hyaline casts. Magnification 150 ×.

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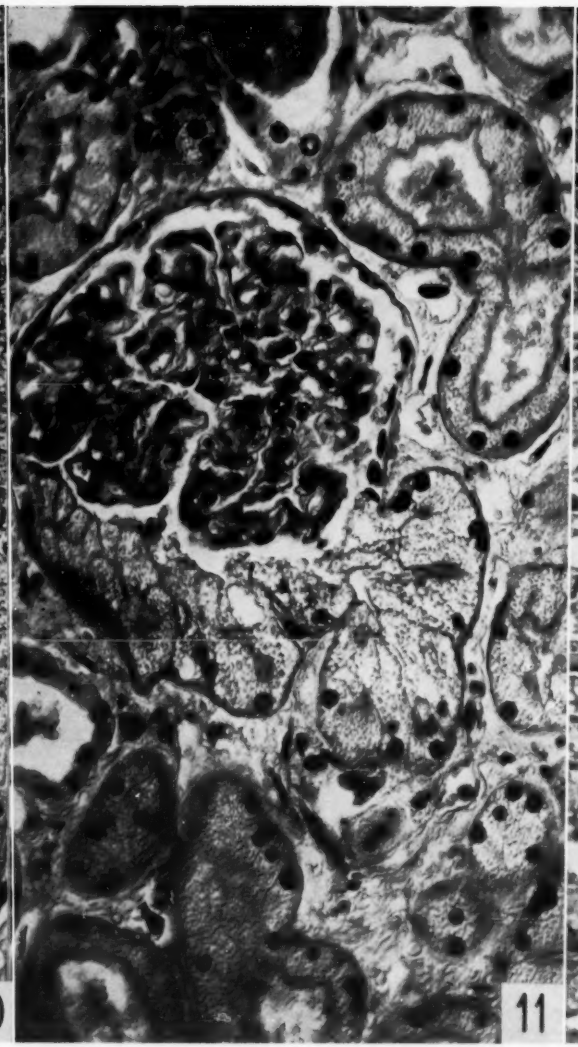
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FIGURE 13

FIG. 13. TERMINAL SEGMENTS OF PROXIMAL CONVOLUTIONS LYING IN THE SUBCORTICAL ZONE OF CONGESTION SHOWING IRREGULAR COMPRESSION AND DISTENTION OF THEIR LUMENS WHICH CONTAIN COAGULATED PROTEINACEOUS MATERIAL

The lining epithelium is either flattened or filled with hyaline droplets. Magnification 500 ×.

FIGURE 14

FIG. 14. CASE 21, DIED ON SIXTH DAY OF PRIMARY SHOCK IN THE HYPOTENSIVE PHASE—HE RECEIVED 5 UNITS OF HUMAN SERUM ALBUMIN IN THE 36 HOURS PRECEDING HIS DEATH

Section of cortex, stained with Gram, showing practically all cross sections of proximal convolutions filled with Gram positive droplets. Magnification 100 ×.

FIGURE 15

FIG. 15. SAME PREPARATION AT HIGHER POWER

Every cross section of proximal convolution in the field is crowded and distended with Gram positive droplets. The nuclei of the epithelial cells, when visible, are well preserved. Magnification 500 ×.

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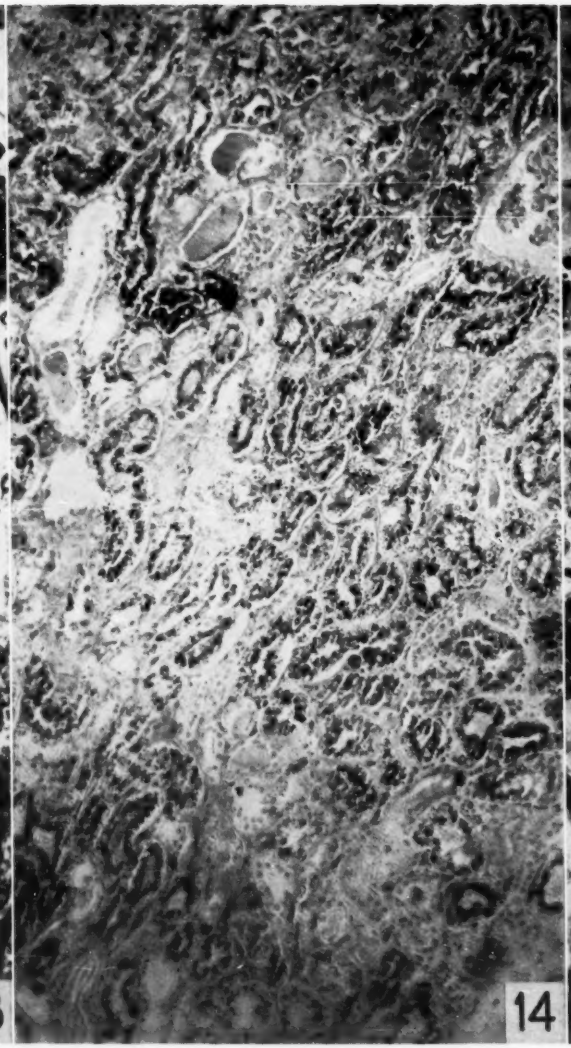

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FIGURE 16

FIG. 16. CASE 39, DIED ON THE SEVENTH DAY OF SHOCK IN THE TRANSITION FROM THE HYPOTENSIVE TO THE OLIGURIC PHASE—ONE OF THE SCATTERED AREAS OF INTERTUBULAR EDEMA IN CORTEX

The epithelium of the proximal convolutions is swollen; a distal convolution contains a solid cast. Magnification 500 ×.

FIGURE 17

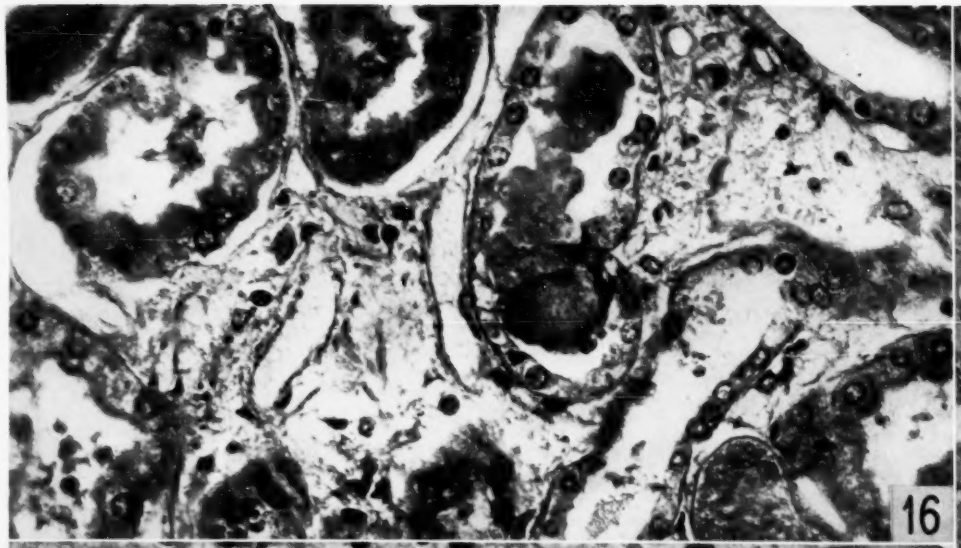
FIG. 17. ENTRAPMENT OF PROTEINACEOUS MATERIAL CONTAINING MANY DESQUAMATED EPITHELIAL CELLS IN "STRAIGHT TUBULES" LYING IN THE SUBCORTICAL ZONE OF CONGESTION

The intertubular capillaries are distended with red cells. Magnification 500 ×.

FIGURE 18

FIG. 18. THE PAPILLA, SHOWING EXTENSIVE EDEMA AND MARKED CONGESTION WITH BEGINNING HEMORRHAGE

The large ducts of Bellini contain the renal failure casts of Addis. Magnification 150 ×.



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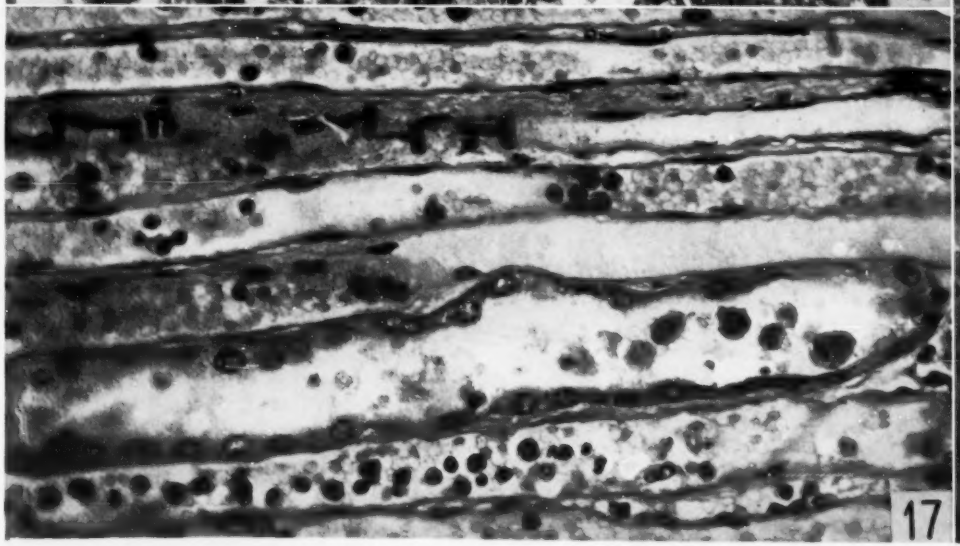

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FIGURE 19

FIG. 19. CASE 28, DIED ON THE TENTH DAY OF TRANSITION SHOCK—THE SUBCORTICAL ZONE OF HEMORRHAGE

No remnants of the intertubular capillaries remain in a mass of crowded red blood cells which isolates and compresses enclosed degenerating and necrotic tubules; above, the remnant of a terminal segment of a proximal convolution; below, less damaged collecting tubules. Magnification 500 ×.

FIGURE 20

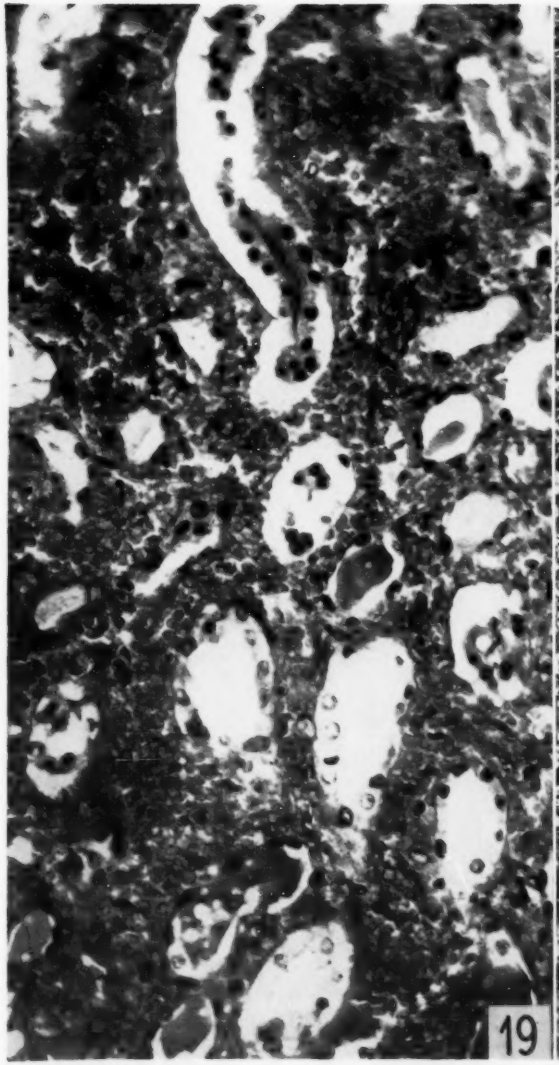
FIG. 20. THE CORTEX, SHOWING IRREGULAR EXTENSION OF THE INTERTUBULAR HEMORRHAGE FROM THE SUBCORTICAL MEDULLA

Proximal convolutions are surrounded and widely separated by the infiltration which reaches in places to the capsule. Magnification 150 ×.

FIGURE 21

FIG. 21. DETAIL OF CORTICAL INTERTUBULAR HEMORRHAGE AND ITS EFFECT ON THE ENCLOSED TUBULES

In the left center three cross sections show the typical appearance of the tubulorhexic lesion in histological section; in each, a portion of the entire wall is destroyed and yet the remaining cells and basement membrane are remarkably well preserved and intact. Magnification 500 ×.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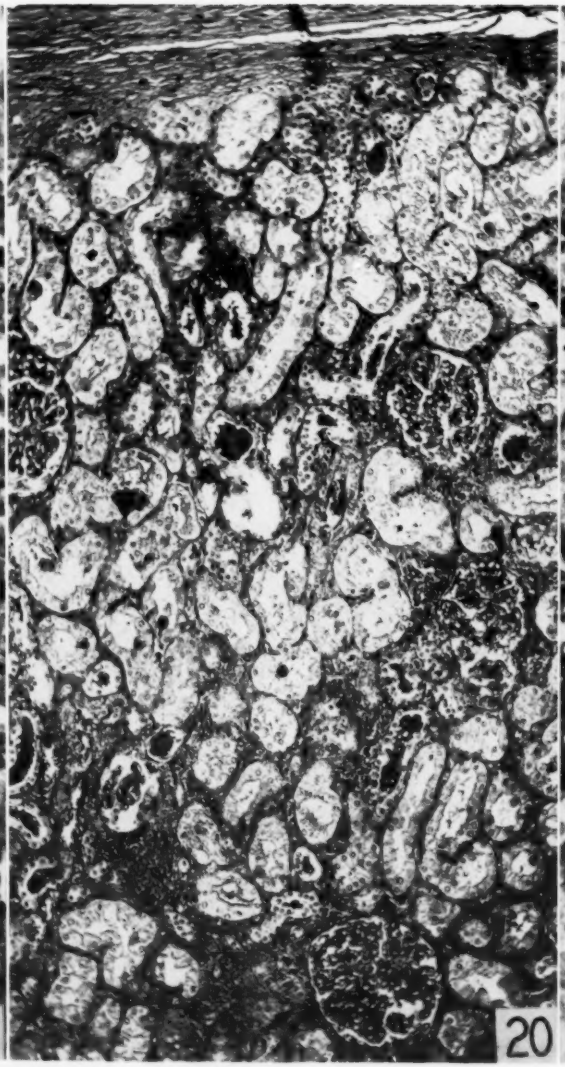
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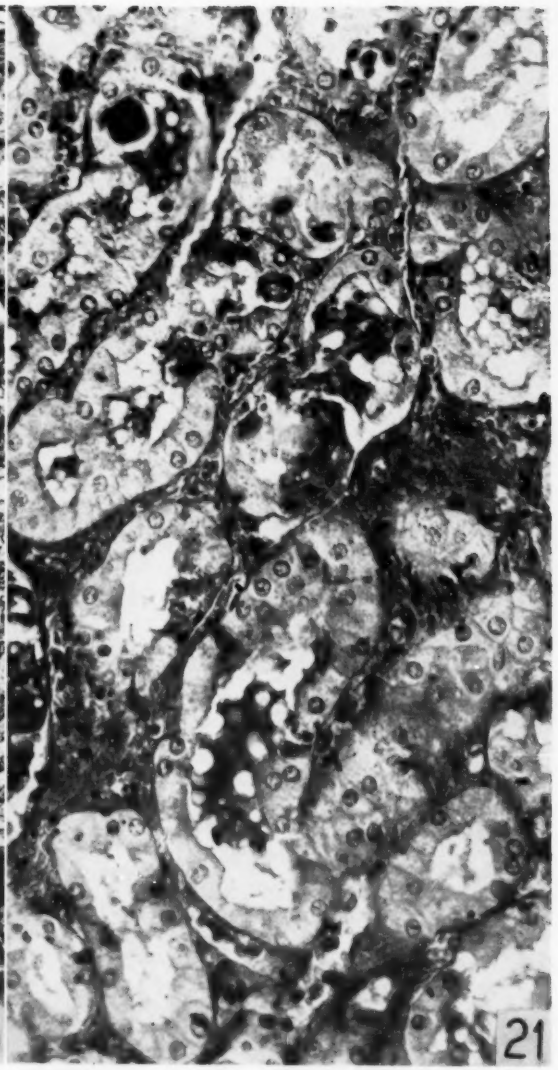
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FIGURE 22

FIG. 22. MID-MEDULLA, SHOWING COMPLETE NECROSIS OF ALL TISSUES INCLUDING TUBULES, MOSTLY COLLECTING, WHICH STILL MAINTAIN THEIR EXTERNAL CONFIGURATION

To the right, stasis in engorged vessels crowded with compacted red cells; there is no extensive hemorrhage. Magnification 150 ×.

FIGURE 23

FIG. 23. THE PAPILLA—EXTENSIVE CONGESTION WITH STASIS AND HEMORRHAGE

The necrotic ducts of Bellini are still recognizable and are filled with huge renal failure casts. Magnification 150 ×.

FIGURE 24

FIG. 24. CASE 31, DIED ON THE ELEVENTH DAY IN THE OLIGURIC PHASE—THE TYPICAL APPEARANCE OF THE CORTEX IN THE STATE OF ESTABLISHED OLIGURIA

Dilatation of all urinary channels above the compression of the zone of intertubular hemorrhage. Bowman's space, proximal convolution and distal convolution (two cross sections contain solid debris) are greatly and evenly distended with resulting pressure effects on tufts and epithelium. Magnification 150 ×.

22

23

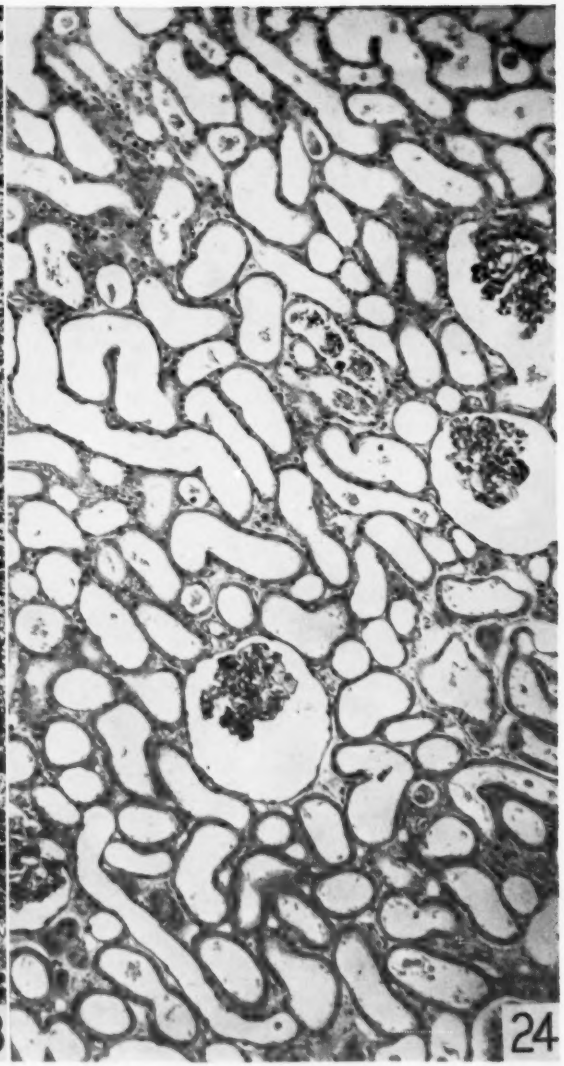
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FIGURE 25

FIG. 25. OUTER STRIPE OF THE OUTER ZONE OF THE MEDULLA

Below, the intertubular hemorrhage is so extreme as to obliterate tubules; above, in the transition to the cortex is seen the beginning dilatation that extends into the cortical tubules shown in Figure 24. Magnification 150 ×.

FIGURE 26

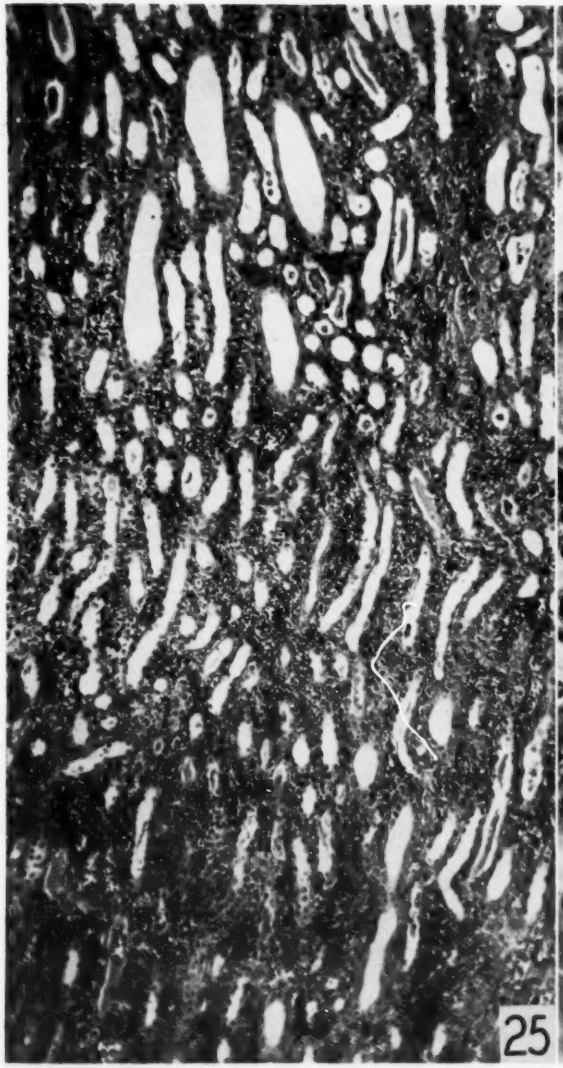
FIG. 26. CASE 25, DIED ON THE TENTH DAY IN THE OLIGURIC PHASE

The large cross section of proximal convolution shows an irregular but living epithelium in which are two mitotic figures. Immediately above, in a smaller cross section, one-half of the tubular wall is composed of atypical regenerated epithelium, the other of invading intertubular connective tissues. Magnification 500 ×.

FIGURE 27

FIG. 27. CASE 41, DIED ON THE TENTH DAY IN THE OLIGURIC PHASE—ONE OF THE SCATTERED AREAS OF CORTICAL INTERTUBULAR EDEMA

The deforming effect of external pressure on the irregular dilatation of proximal convolutions is apparent. Magnification 500 ×.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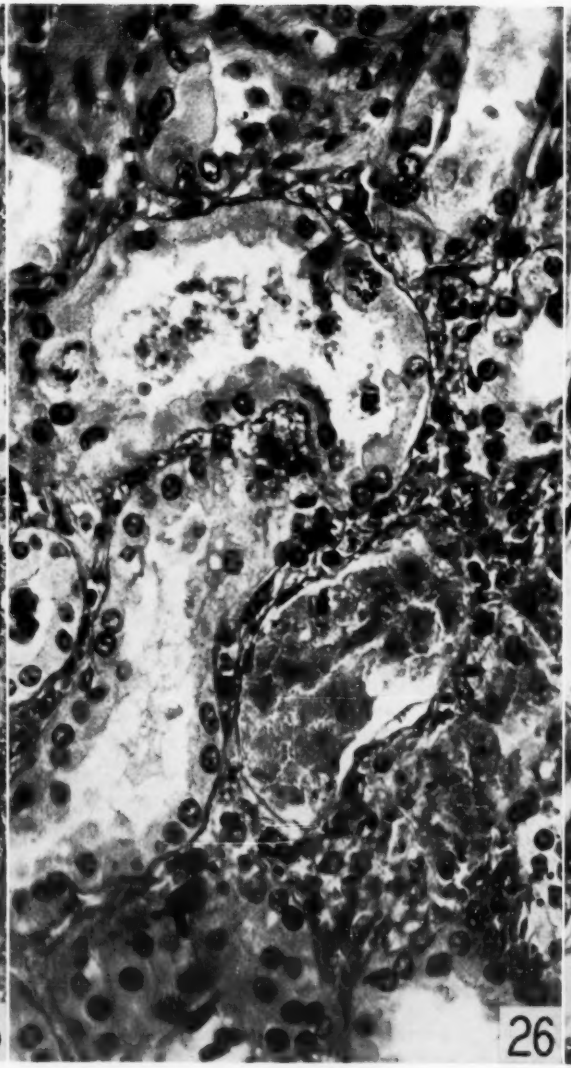
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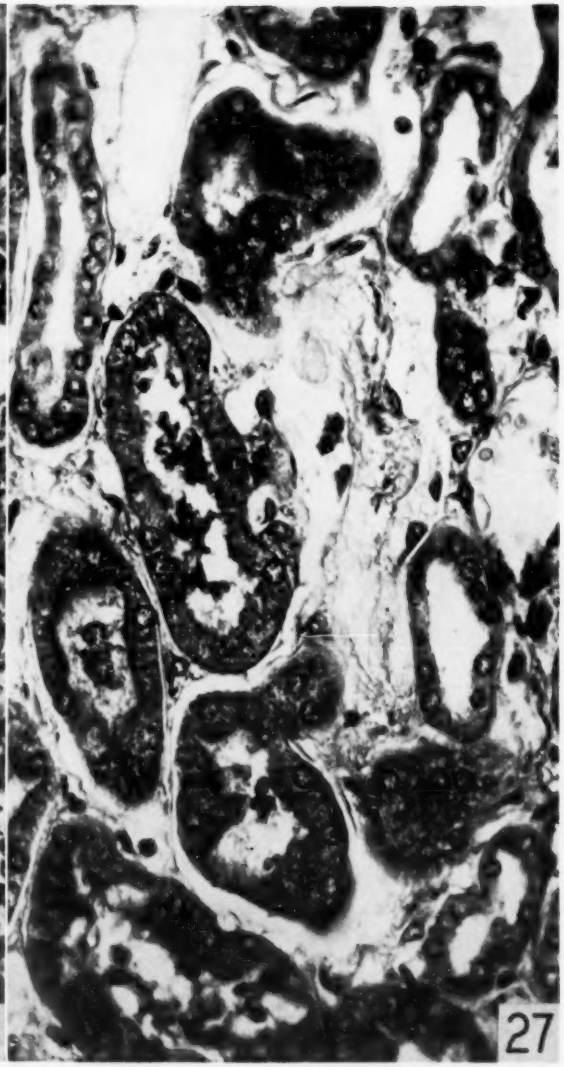
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FIGURE 28

FIG. 28. CASE D, DIED ON THE FIFTEENTH DAY IN TRANSITION FROM THE OLIGURIC TO THE DIURETIC PHASE—DIFFUSE HEMORRHAGIC INFILTRATION THROUGHOUT THE PAPILLA

The contours and epithelial cells of the ducts of Bellini are still intact. Magnification 150 ×.

FIGURE 29

FIG. 29. OUTER ZONE OF THE MEDULLA

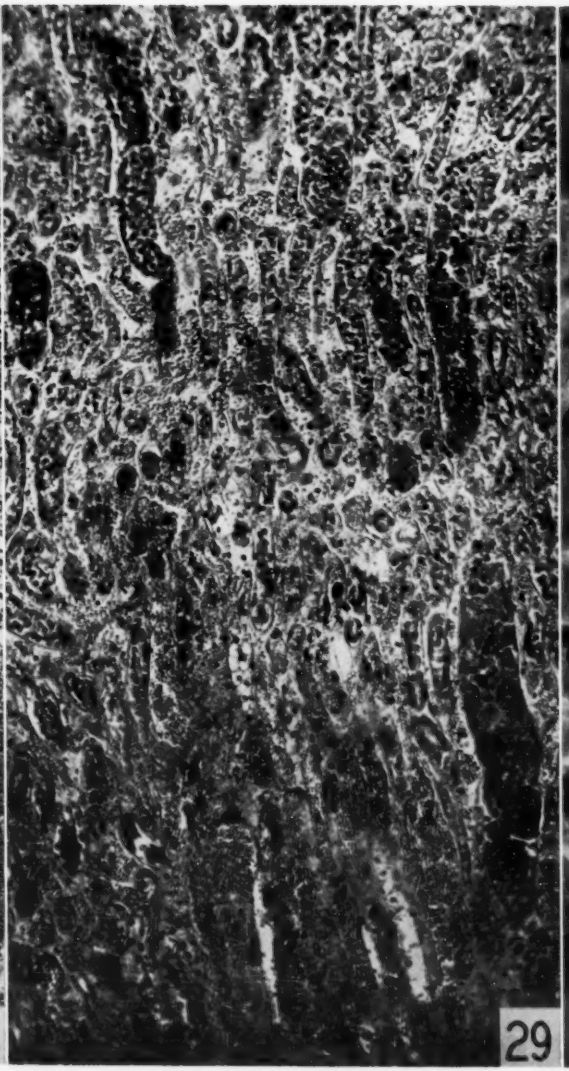
The external configuration of collecting tubules still persists but there is complete necrosis of all tissues. Magnification 150 ×.

FIGURE 30

FIG. 30. OUTER STRIPE OF OUTER ZONE SHOWING SUBCORTICAL AREA OF NECROSIS AND HEMORRHAGE

Extending down from the cortex are completely necrotic terminal segments of proximal convolutions. Magnification 500 ×.

28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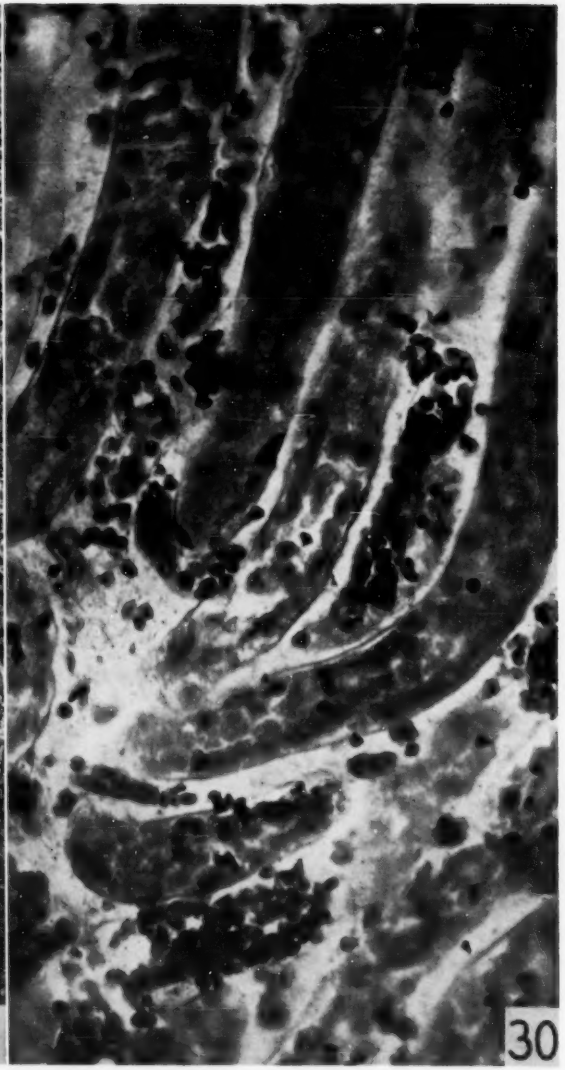
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FIGURE 31

FIG. 31. SOMEWHAT DEEPER IN THE MEDULLA

In the upper left, necrotic terminal segments of proximal convolutions; below, wisp-like terminal segments with huge hyperchromatic giant-cell nuclei extend into the region of intertubular hemorrhage. Magnification 500 ×.

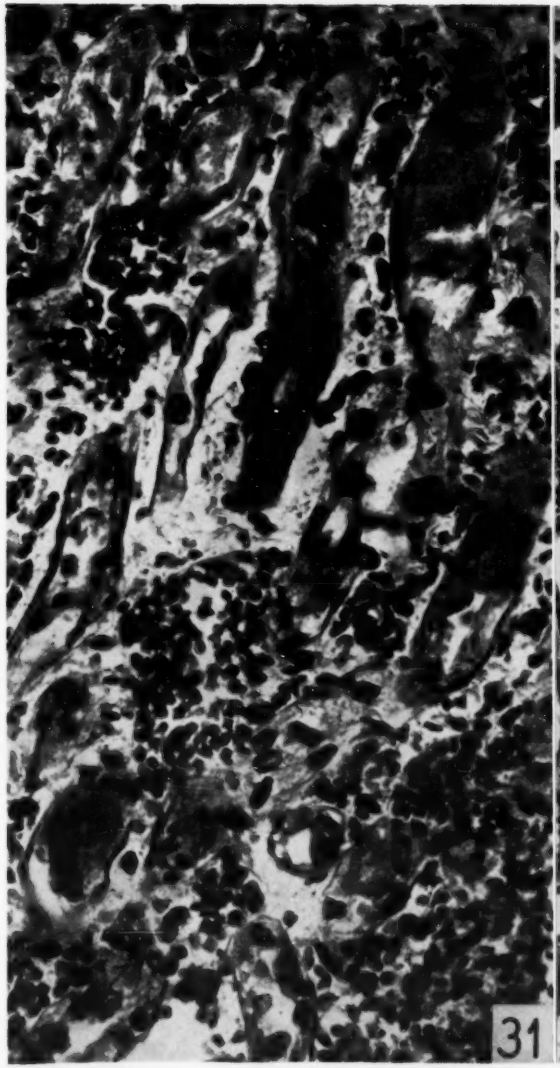
FIGURE 32

FIG. 32. CORTEX SHOWING THE PERSISTING OLIGURIC DILATATION OF CONVOLUTIONS, PROXIMAL AND DISTAL, AND OF BOWMAN'S SPACE—MAGNIFICATION 150 ×

FIGURE 33

FIG. 33. HIGHER POWER VIEW OF SAME DILATED CORTICAL PROXIMAL CONVOLUTIONS

The epithelium shows the oval and irregular nuclei of flattened regenerated cells which have replaced the original epithelium which had been destroyed by necrosis and desquamation. Magnification 500 ×.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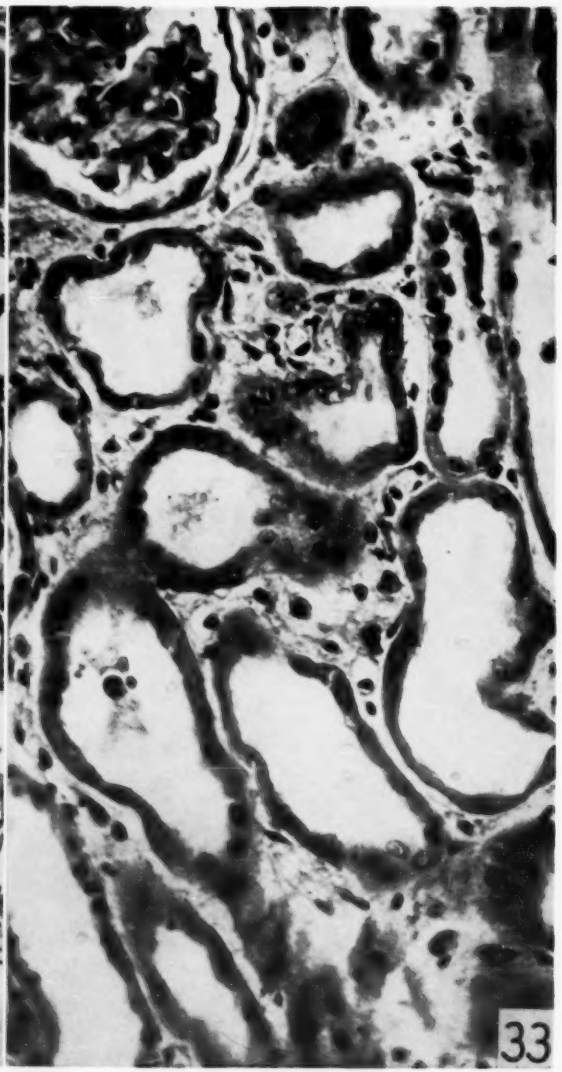
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FIGURE 34

FIG. 34. CASE 43, DIED ON THE SIXTEENTH DAY OF HIS ILLNESS AND IN THE FIFTH DAY OF THE DIURETIC PHASE—OUTER CORTEX, CAPSULAR SURFACE TO LEFT

The proximal convolutions are lined with epithelium of their original normal type and the dilatation of the Oliguric Phase has disappeared. The appearance suggests that not much tubular damage had occurred at any previous phase of the disease. Magnification 150 ×.

FIGURE 35

FIG. 35. OUTER STRIPE OF OUTER ZONE OF MEDULLA

Extending down from the cortex are the terminal segments of proximal convolutions. They are lined with a deeply staining atypical regenerated epithelium. The destruction of the original cells of these tubules was the result of the intertubular hemorrhage in this region (*cf.* Figure 30); this hemorrhage is no longer apparent and the intertubular spaces are filled with fibroblastic tissue. Magnification 350 ×.

FIGURE 36

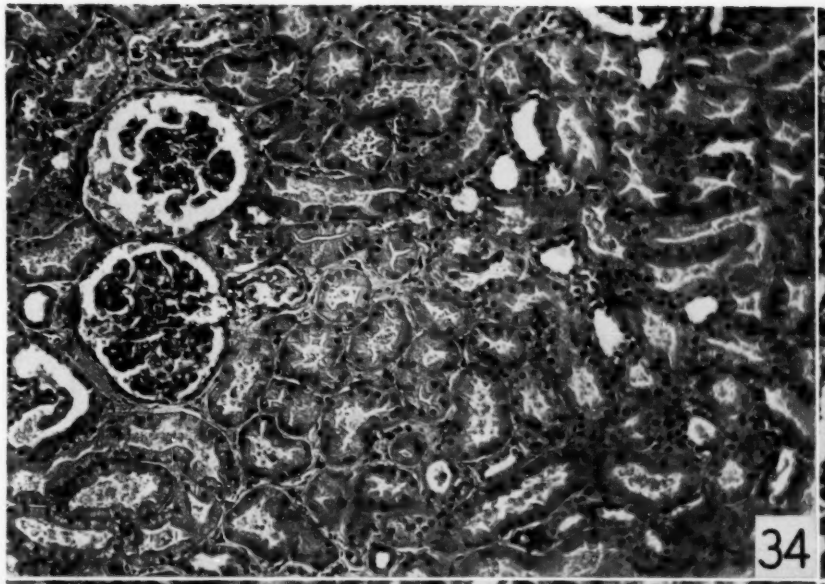
FIG. 36. A DEEPER LEVEL IN THE MEDULLA

Among areas of persisting intertubular hemorrhage ill-defined patches of resolution are seen where, between atypical hyperplastic tubules, mostly collecting, a fibroblastic connective tissue has replaced earlier hemorrhage. Magnification 150 ×.

FIGURE 37

FIG. 37. OUTER MEDULLA

The Masson stain shows the increase in fine collagenous fibrils between tubules in an area of resolution with thickening of the basement membranes of tubules. Magnification 350 ×.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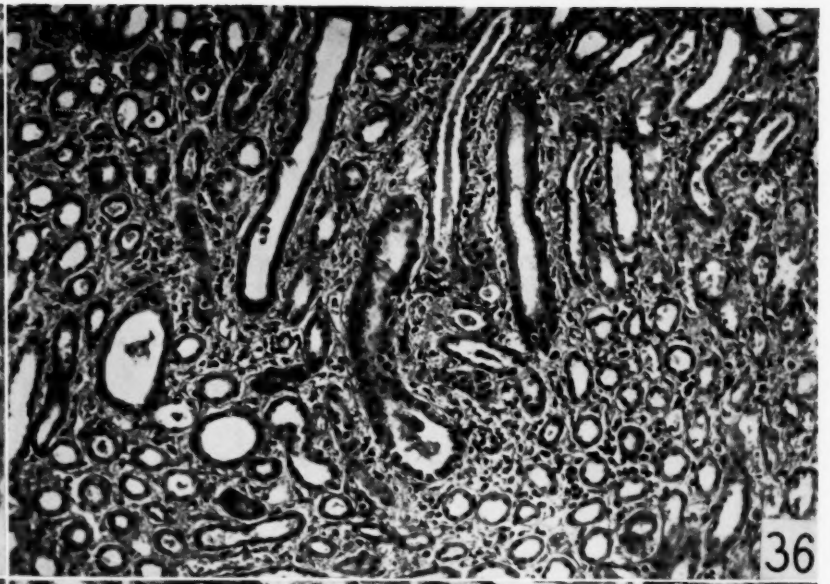
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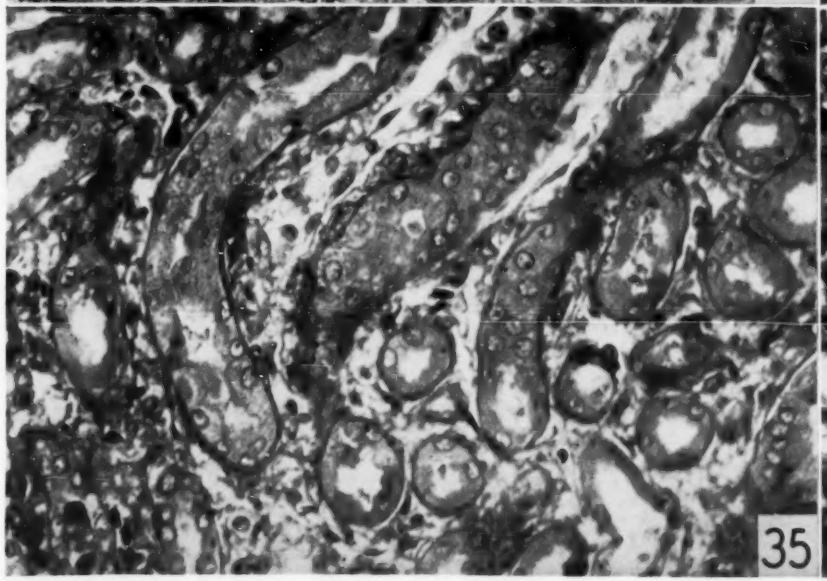
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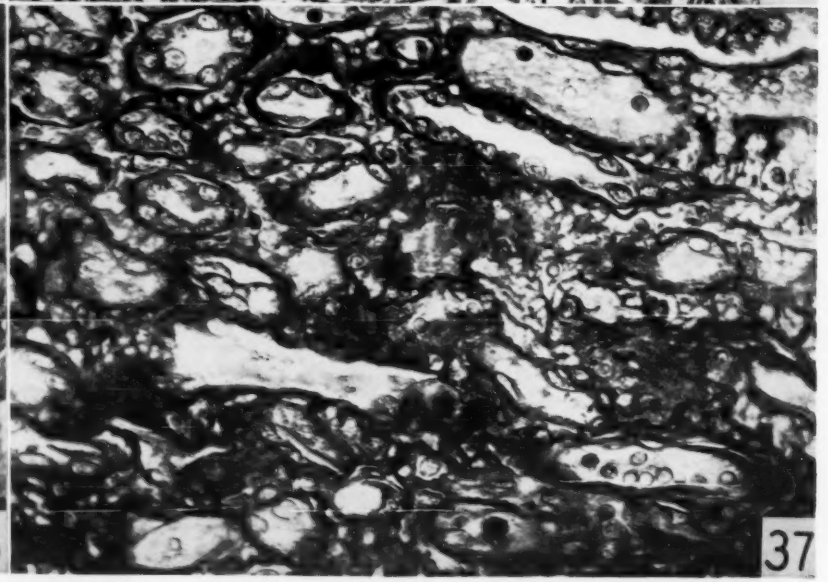
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FIGURE 38

FIG. 38. AN EXTENSION OF FIBROUS CONNECTIVE TISSUE FROM THE SUBCORTICAL MEDULLA INTO THE LOWER CORTEX

As seen in Figure 34, the greater part of the cortex was free of any evidence of damage. Magnification 150 ×.

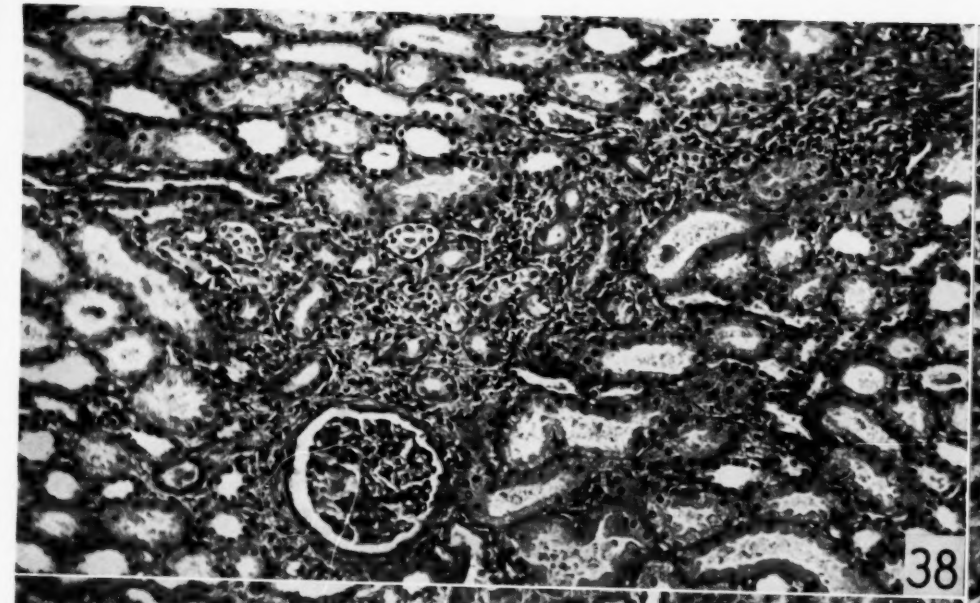
FIGURE 39

FIG. 39. CASE 19, DIED ON THE EIGHTEENTH DAY OF THE DISEASE AND ON THE NINTH DAY OF THE DIURETIC PHASE—AN AREA OF RESOLVED HEMORRHAGE IN THE OUTER MEDULLA SHOWING THE INCREASE IN FIBROBLASTIC CONNECTIVE TISSUE BETWEEN DISTORTED TUBULES—MAGNIFICATION 150 ×

FIGURE 40

FIG. 40. CASE 33, DIED ON THE NINETEENTH DAY OF THE DISEASE AND THE TENTH DAY OF THE DIURETIC PHASE—MID-CORTEX

Proximal convolutions are lined with a low and irregular layer of hyperplastic atypical epithelial cells. Although the lumen is consequently wide, there is no evidence of thinning of their walls from the tension of dilatation such as occurred in the Oliguric Phase (*cf.* Figure 24). Magnification 500 ×.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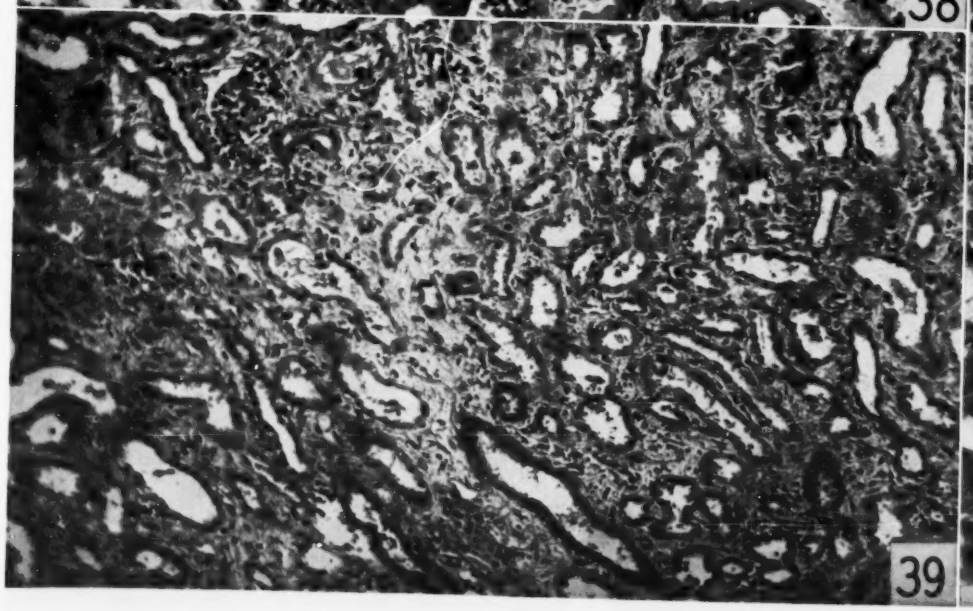
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FIGURE 41

FIG. 41. MID-MEDULLA SHOWING AN AREA OF HEMORRHAGE SURROUNDING COLLECTING TUBULES WHICH ARE IRREGULARLY DILATED AND DISTORTED IN OUTLINE BY A MARKEDLY HYPERPLASTIC PROLIFERATION OF THEIR EPITHELIUM—MAGNIFICATION 300 ×

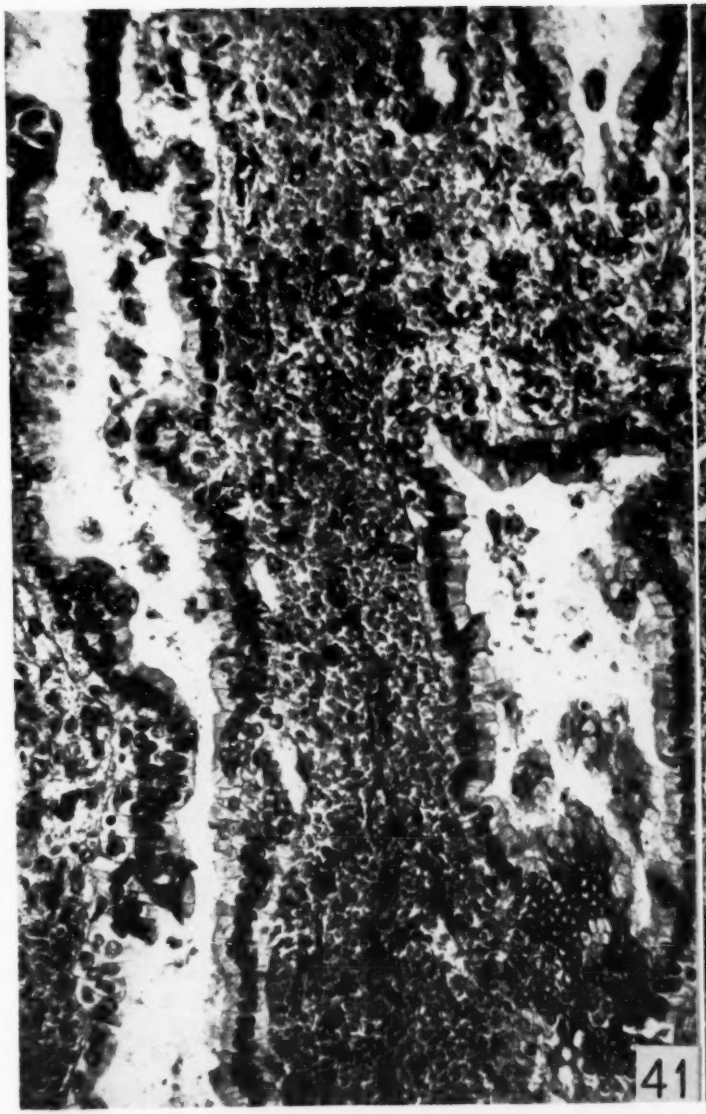
FIGURE 42

FIG. 42. CASE E, DIED OF CEREBRAL COMPLICATIONS 149 DAYS AFTER THE ONSET OF EHF—THE OUTER MEDULLA SHOWING AN AREA OF MODERATE FIBROSIS IN WHICH COLLECTING TUBULES ARE SEEN WITH A REDUNDANT HYPERPLASTIC EPITHELIUM—MAGNIFICATION 150 ×

FIGURE 43

FIG. 43. OUTER CORTEX

Both kidneys showed surface scarring which was more marked in the left. A long wedge-shaped fibrous scar extends from the mid-medulla to the capsule in which there is a heavy round cell infiltration and atrophy of enclosed glomeruli and tubules. Magnification 150 ×.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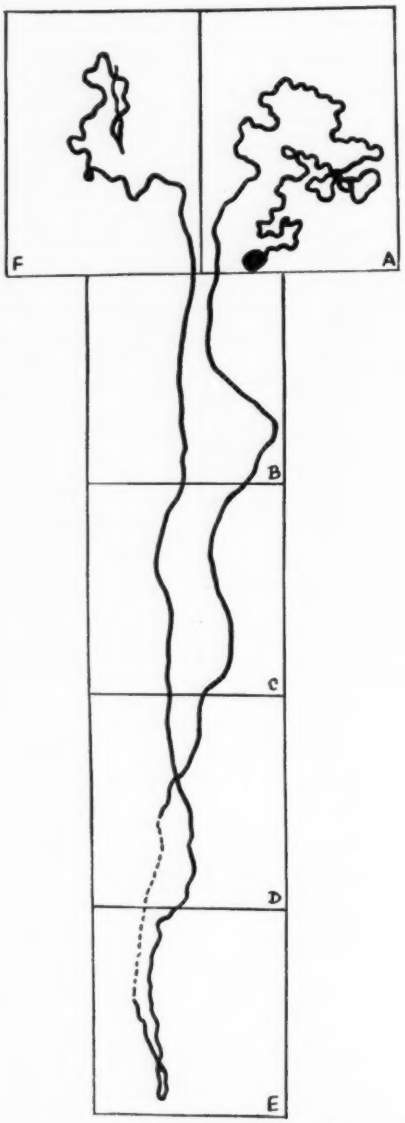
43

PLATE I (A TO F)



A complete nephron from Case 38. The orientation of the plates is shown in the line tracing. The alterations are so slight that this plate may be taken as showing, except where noted, the appearance of a normal dissected nephron stained with iron hematoxylin.

IA—The nuclei do not stain and so appear as clear round objects; the mitochondrial substance stains heavily, the rodlets and granules, individually invisible due to the thickness of the tubule, obscure the nuclei in the upper convolutions where, in conformity to the normal gradient, they are most concentrated. The glomerulus, dense black due to the mass of its tissue, is normal in configuration; the outline of the tuft within the capsule is faintly visible. Original magnification 200 ×, here reduced to 80 ×.

Plate IB

To the right the terminal segment of the proximal convolution. At *a*, it enters the subcortical zone of congestion. It is slightly compressed and as a result there is some irregularity of staining. To the left, the ascending limb is moderately dilated and consequently its irregularly thinned wall stains variably.

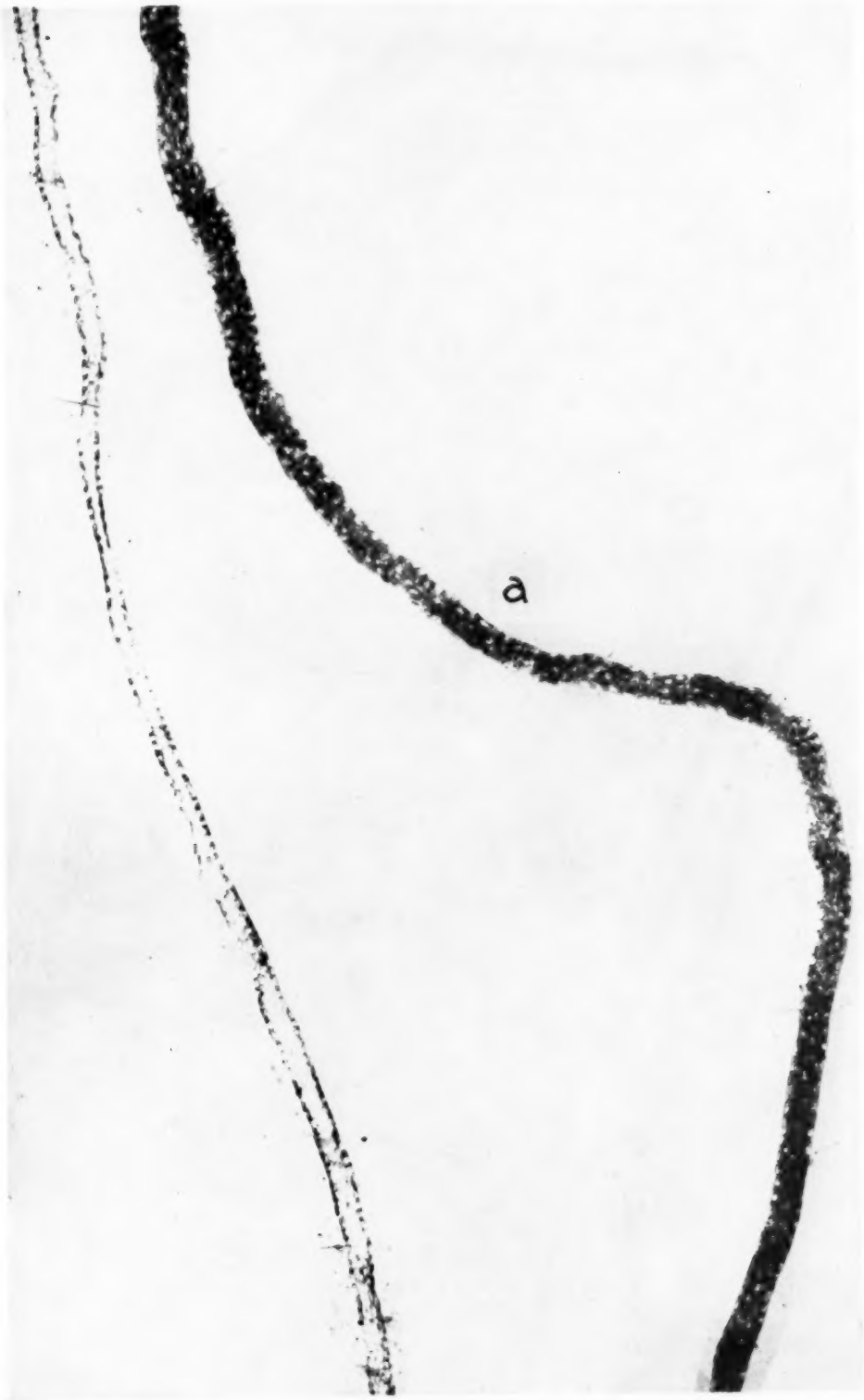

a

PLATE IC

As in IB, the middle third of the terminal segment of the proximal convolution appears more dense than normal due to the pressure of surrounding intertubular congestion.

PLATE ID

The termination of the proximal convolution to left; the capillary-like thin portion of Henle's loop was swept away during staining.

Plate IE

The loop of Henle; a remnant of the narrow portion remains and passes into the thick portion, which, still descending, turns through the loop into the broad ascending limb visible in ID, C, and B. Two small artifactual breaks are present near the loop.

PLATE IF

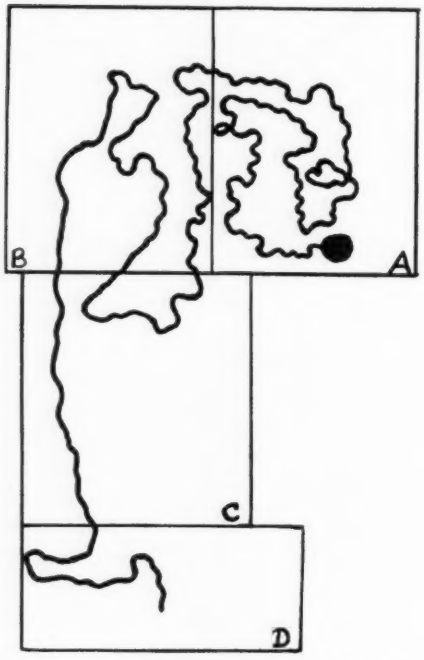
Passage of the thick portion of the ascending limb into the distal convolution which lay intertwined in the proximal convolutions of IA. Except for an irregular dilatation (*cf.* Figure 1) which produces variable staining of its thin wall, the distal convolution is empty and normal in configuration. From the top of the plate the connecting tubule, not dilated, joins the origin of a peripheral collecting tubule.

PLATE II. PORTIONS OF TERMINAL MEDULLARY SEGMENTS OF PROXIMAL CONVOLUTION WHICH LAY IN THE SUBCORTICAL ZONE

To the left can be seen the essentially normal cellular pattern of the terminal segment from the nephron of Case 38 shown in Plate I. Center, from Case 21, the tubule was surrounded by intertubular congestion. The cellular pattern is no longer visible for the compressed cells are filled with deeply stained hyaline droplets. To the right, a similar tubule from the same case. Droplets are seen throughout; in places the cell outlines filled with droplets can still be seen. At *a* the lumen is distended and filled with homogeneous lightly stained proteinaceous material. For appearance in histological section of a similar lesion, *cf.* Figure 13. Original magnification of 200 × is here reproduced.

a

PLATE III (A TO D)



All that remained of a proximal convolution from Case 28; for orientation, see outline tracing.

IIIA. The irregularity of the glomerular contours is due to pressure of the cover glass; its configuration was normal. Beginning in the first loops of the convolution are irregular stretches of tubule (↑) showing the typical tubulorhexic disruption of anoxia; between these the tubule is better preserved. (Compare with Figures 20, 21, and contrast with Plate IA.) Original magnification of 200 ×, is here reduced to 80 ×.

PLATE IIIB

To the right, a loop of cortical proximal convolution which entered the zone of subcortical congestion and hemorrhage at *a*; to the left, loops of its medullary continuation show increasing disruptive damage.

a

PLATE IIIC. TERMINAL MEDULLARY PORTION OF PROXIMAL CONVOLUTION LYING IN THE ZONE OF HEMORRHAGE

The entire extent of the tubule is almost continuously necrotic.

PLATE IIID

The terminal medullary segment fades into a wisp of necrotic tubule.

PLATE IV. A LOOP OF HENLE FROM THE SAME CASE LYING IN THE SUBCORTICAL ZONE OF HEMORRHAGE

Scattered through the length of fairly intact tubule are seen the tubulorhexic lesions of renal ischemia. At *a*, the bend of the loop, the lumen is filled with solid material. Original magnification of 100 × is here reduced to 40 ×.

a

PLATE V. COLLECTING TUBULES OF THE SAME CASE LYING IN THE ZONE OF HEMORRHAGE IN THE OUTER STRIPE OF THE OUTER ZONE OF THE MEDULLA

All show extensive segments of complete tubular necrosis which, with their black stained content of coagulated debris, produces a marked irregularity in tubular outline. At left, the luminal content is a solid cast, to the right, scattered debris and desquamated cells are visible. Original magnification of 100 × is here reduced to 40 and 20 ×.

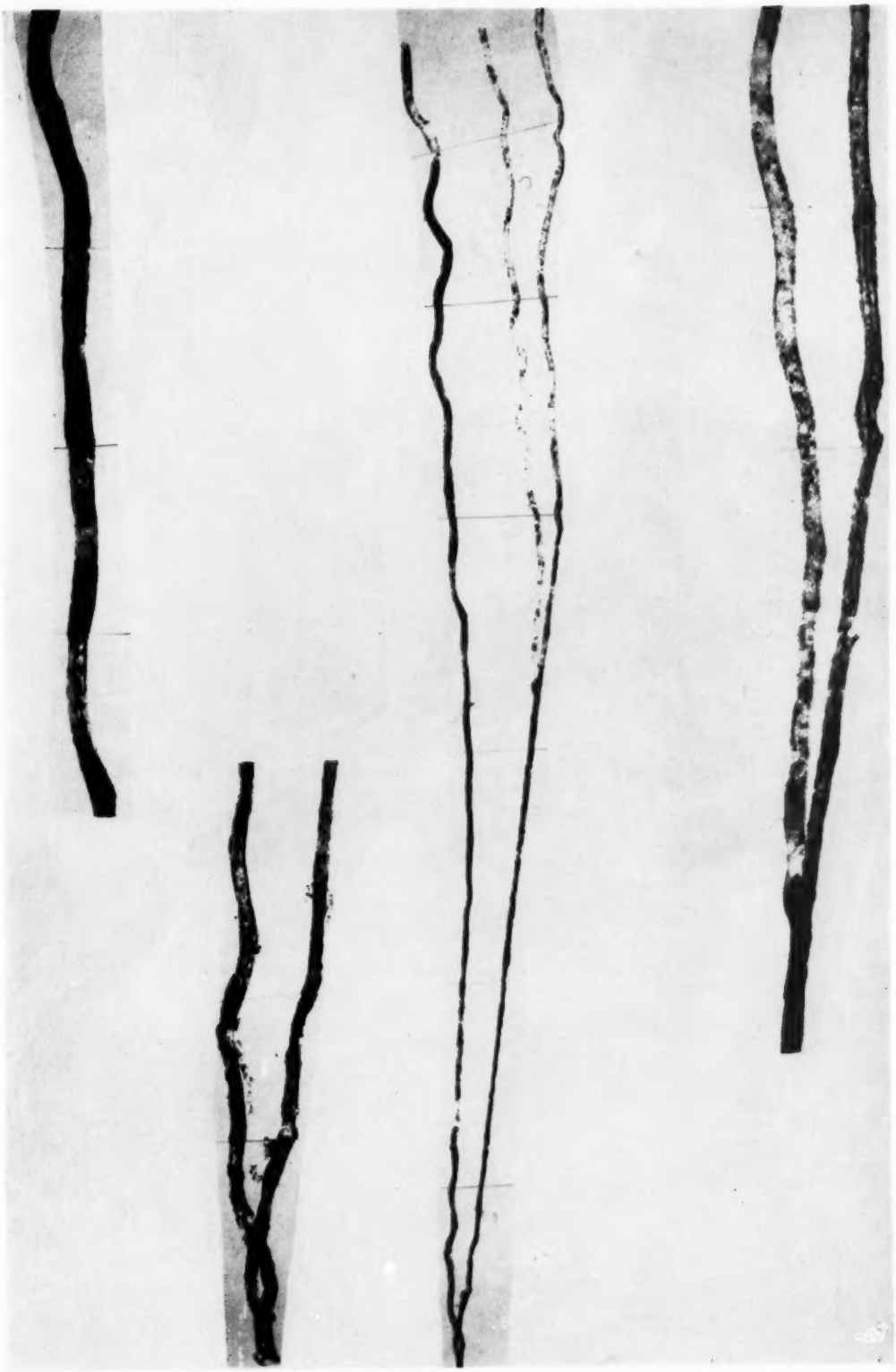
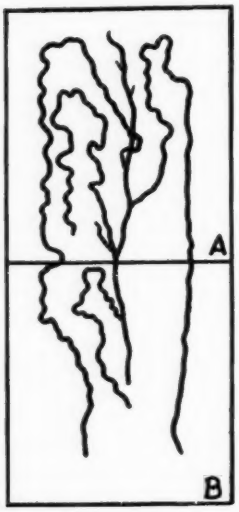


PLATE VI (A TO B)



Orientation of plates shown in outline tracing. From the same case; the formation of the peripheral collecting system by the junction of the connecting tubules of 4 nephrons; 4 others have been removed, leaving only their connecting tubules attached.

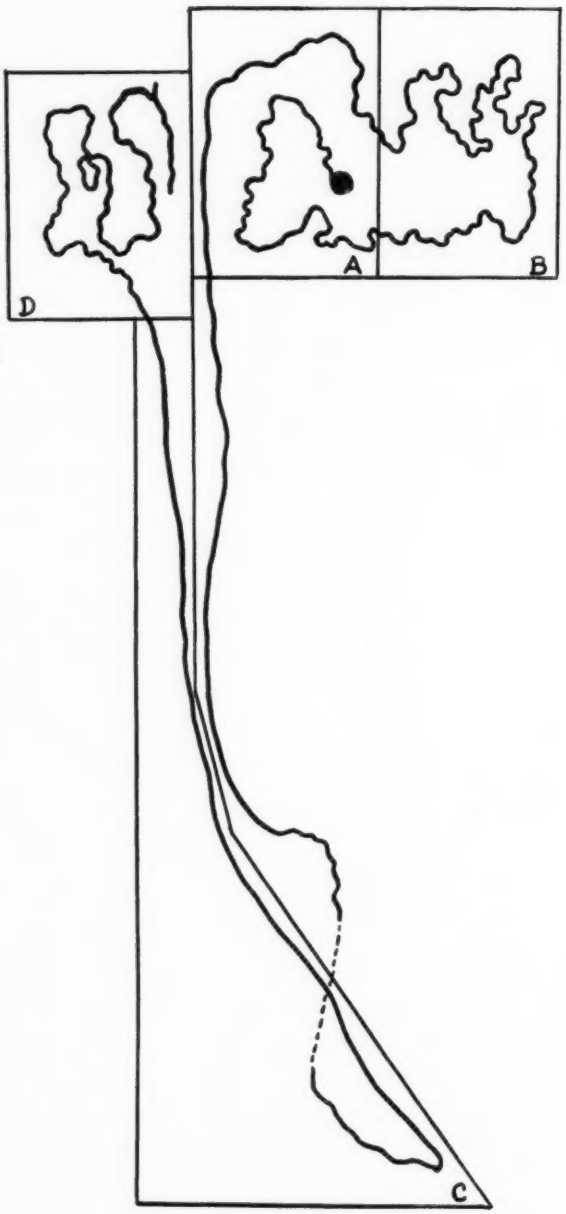
VIA. Three distal convolutions (d) filled with coagulum, which stains dense black and so obscures the tubule wall, are moderately dilated. The connecting tubules (c) are clear of obstruction, but the central collecting tubule is solid with obstructing material. Note that all these tubules, though filled with coagulum, are relatively well preserved as compared to the proximal convolution of Plate III, thus showing the typical distribution of the lesions of ischemia in all forms of Acute Renal Failure. Original magnification of 100 × is here reduced to 40 ×.

d
c
d
c
d
c

PLATE VIB. CONTINUATION OF TUBULES OF VIA

Note the dilatation of the ascending limbs on the left that lead to occluded distals. The collecting tubule in the center is filled with deeply stained material.

PLATE VII (A TO D)



Orientation of plates shown in outline tracing. A nephron from Case 31 in the Phase of Established Oliguria showing dilation throughout its length.

PLATE VIIA. THE CORTICAL PORTION OF THE PROXIMAL CONVOLUTION

Bowman's space is distended and the tuft compressed. Note larger afferent arteriole entering the "polkissen" and a stub of the narrower efferent. After the first coil of proximal convolution the tubule is irregularly distended with resultant patchy thinning of its wall. Original magnification of 200 × is here reduced to 80 ×.

PLATE. VIIB. CONTINUATION OF DILATED CORTICAL PROXIMAL CONVOLUTION

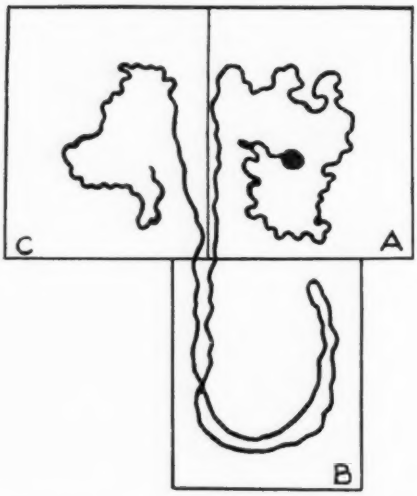
Note the irregular thinning of the epithelial pattern in the distended portions.

PLATE VIIC. PORTIONS OF DILATED ASCENDING LIMB AND LOOP

The tubule is well preserved in spite of distention.

PLATE VIID. THE DILATED DISTAL CONVOLUTION, CONTAINING SOME DARK STAINING DEBRIS, THE CONNECTING TUBULE AND COLLECTING TUBULE ALL WELL PRESERVED

PLATE VIII (A TO C)



The tracing shows the orientation of the three plates of a complete cortical short-looped nephron from Case D, who died in the period of transition from oliguria to diuresis.

Plate VIIIA. The glomerulus is of normal configuration; the dark spot on the afferent arteriole is a collection of "myo-epithelial" cells of the juxta-glomerular apparatus. The greater part of the proximal convolution is dilated. As a result of atypical regeneration the epithelium of its wall is irregular in thickness, and redundant. For histological appearance *cf.* Figures 32 and 33. At *a* there is an incompletely healed tubulorhexic lesion. There are many desquamated epithelial cells in the lumen of the convolution shown to the left. Original magnification of 200 × is here reduced to 80 ×.

a

PLATE VIIIB

To the right the continuation of the proximal convolution which, dilated and containing desquamated epithelial cells, ends at *a*. At *b* the thin portion of the loop passes to the thicker portion and through the loop ascends toward the distal convolution.

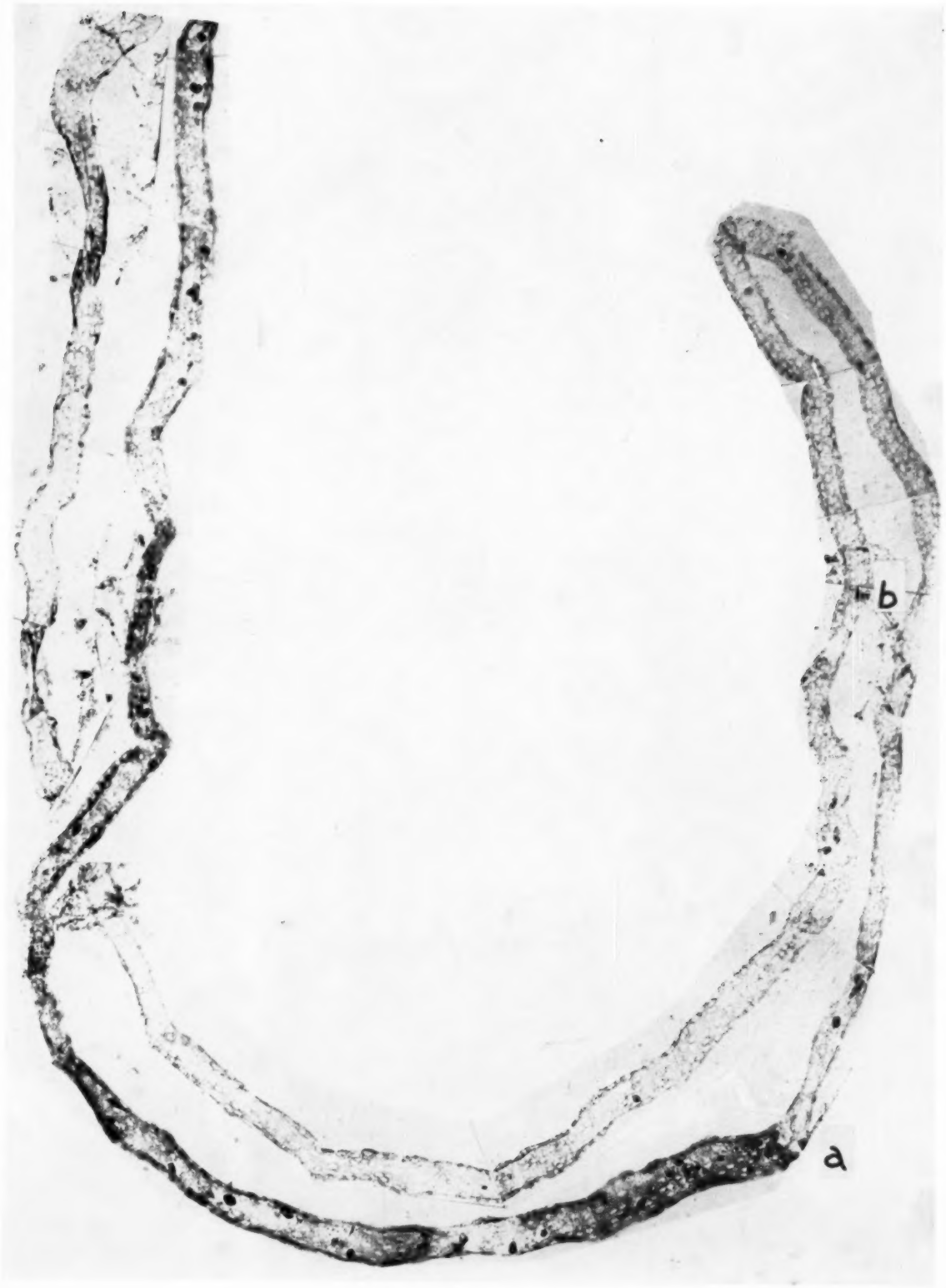
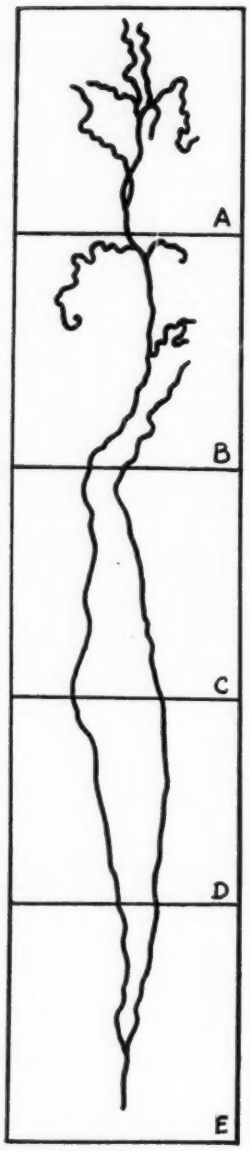

b
a

PLATE VIIIC. THE IRREGULARLY DILATED DISTAL CONVOLUTION WITH THINNED WALL.

The linear marks at *a* are folds that developed as the greatly distended tubule collapsed after it was dissected. At *b* the connecting tubule, less distended, contains deeply stained coagulated material. Note that the epithelium covering the coagulated mass is thin but well preserved.

a
b

PLATE IX (A TO E)



From the same case. Orientation of plates in line tracing.

PLATE IXA. ORIGIN OF THE PERIPHERAL COLLECTING TUBULE SYSTEM

Five connecting tubules, all filled with deeply stained coagulated material, but intact, which lay beneath the capsule in the outer cortex. The cellular pattern of the collecting tubule is normal and it is not filled with coagulum. Original magnification of 200 × is here reduced to 80 ×.

PLATE IXB. THE SAME COLLECTING TUBULE IN MID-CORTEX

Three more connecting tubules join the main tubule which, as shown by its clear cellular pattern, is intact and empty. To the lower right, a neighboring collecting tubule.

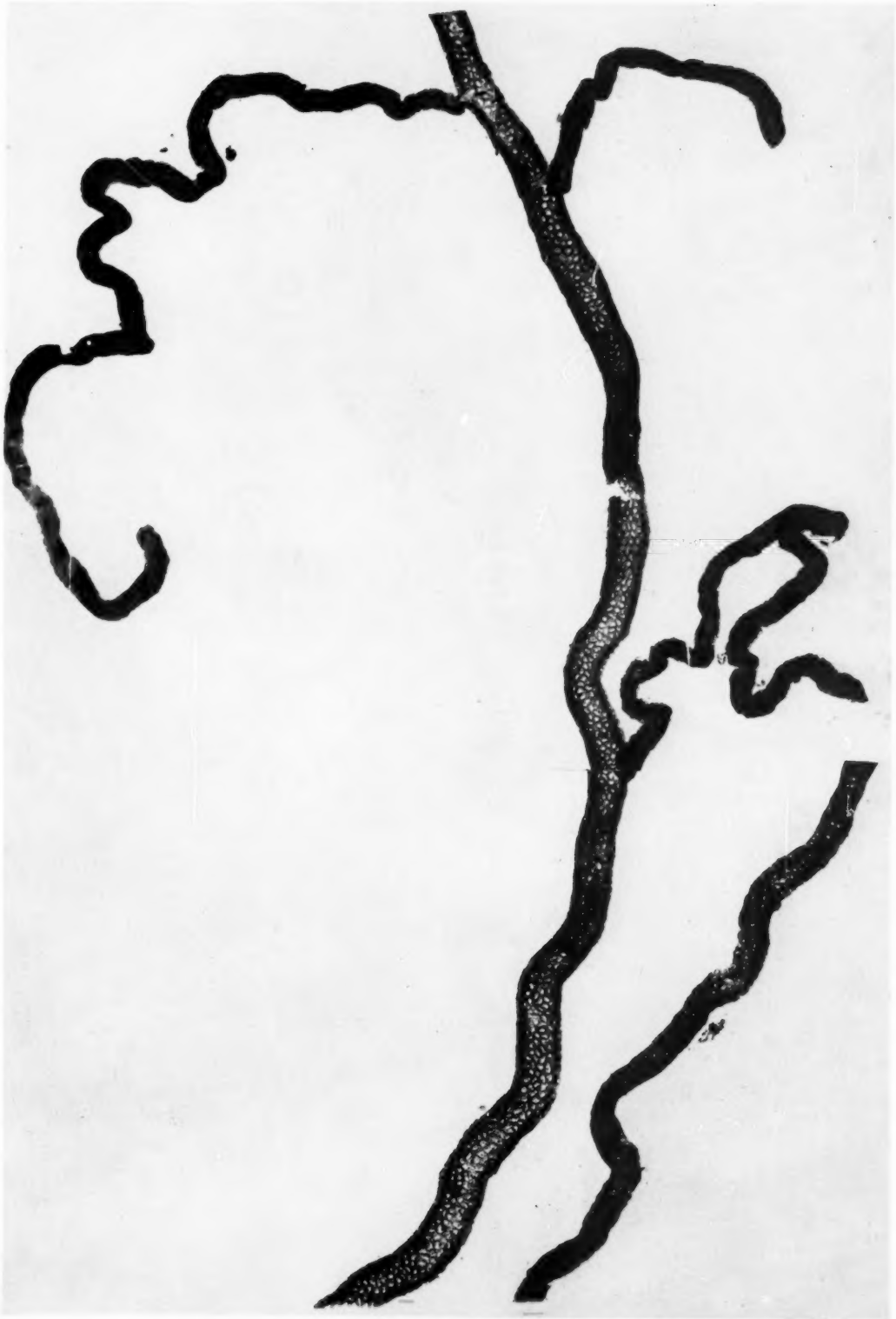


PLATE IXC. CONTINUATION OF THE TWO COLLECTING TUBULES INTO THE SUBCORTICAL AREA OF HEMORRHAGE IN THE OUTER ZONE OF THE MEDULLA

Below the level *a*, the damage to the epithelial cells is barely apparent in the loss of clarity of the nuclear pattern in the tubule to the left (*cf.* see Plate IXB), and is obvious in that to the right.

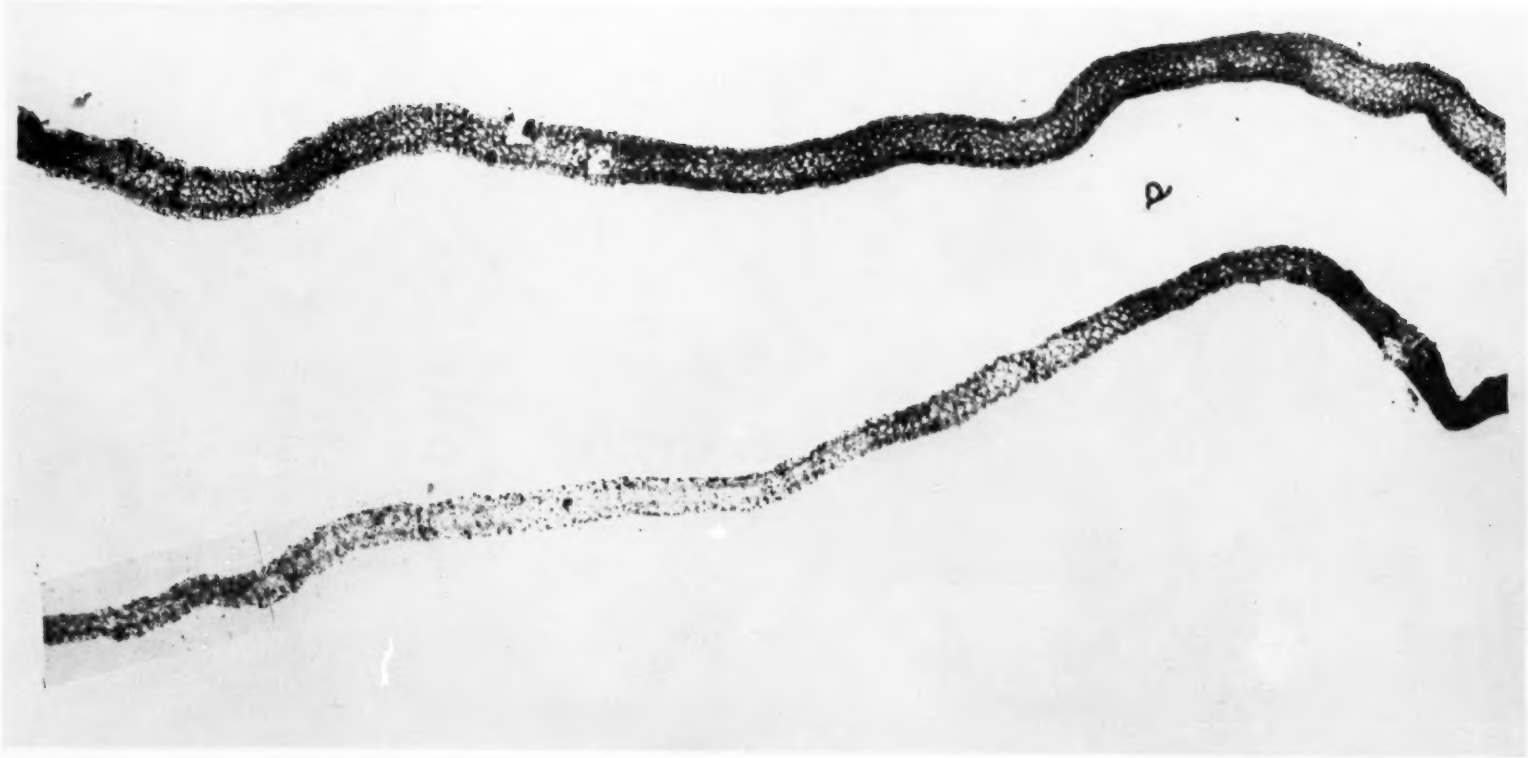


PLATE IXD. CONTINUATION OF THE TWO COLLECTING TUBULES WITH EXTENSIVE EPITHELIAL NECROSIS OF THE GREATER PART OF BOTH

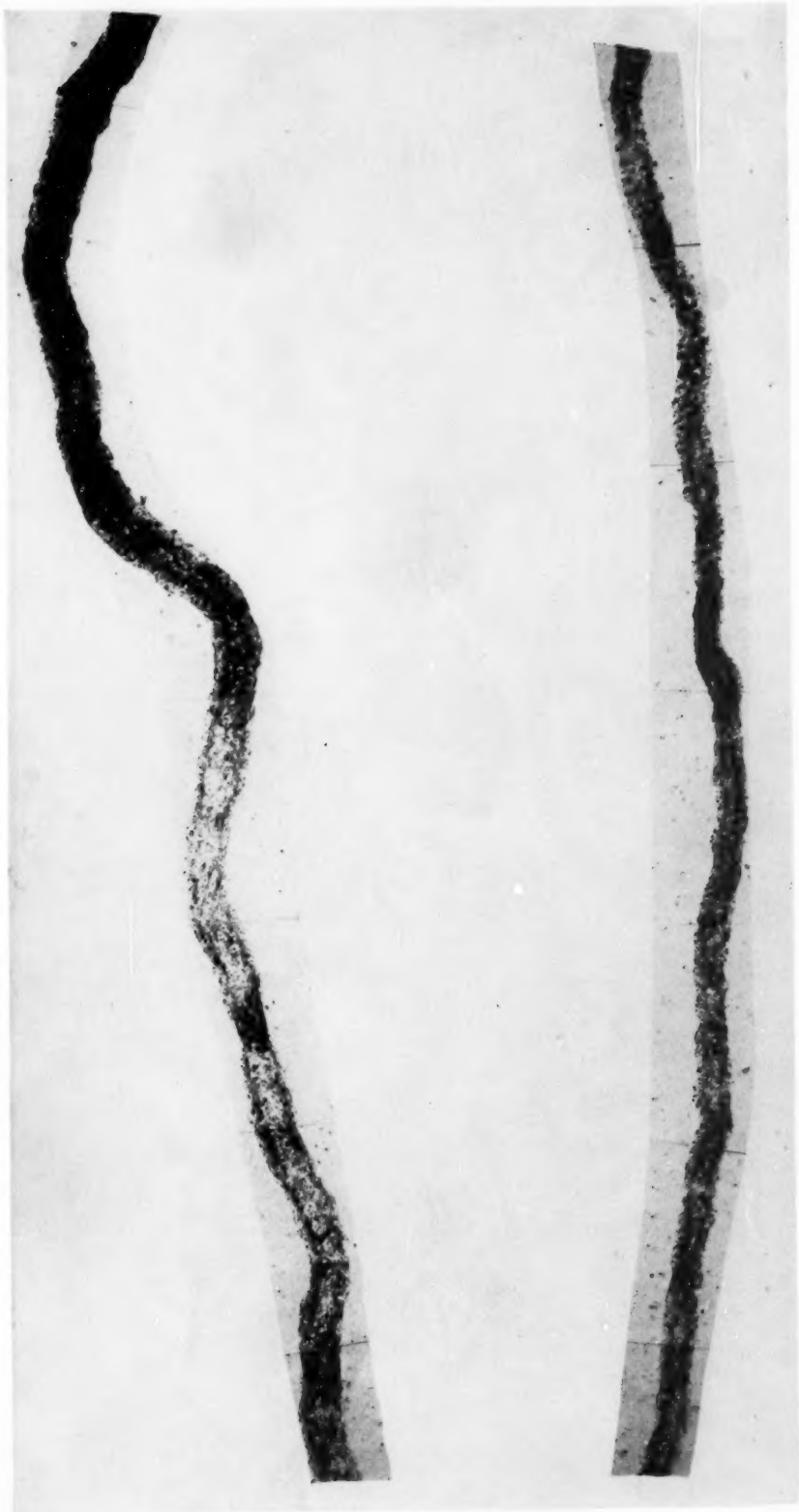


PLATE IXE. CONTINUATION OF THE TWO COLLECTING TUBULES INCLUDING THEIR JUNCTION IN MID-MEDULLA

Only the external configuration of the two tubules, now entirely necrotic, remains. After the tuning-fork junction the tubule continues, necrotic and filled with deeply stained debris. For histological appearance of similar necrotic collecting tubules, *cf.* Figure 29.

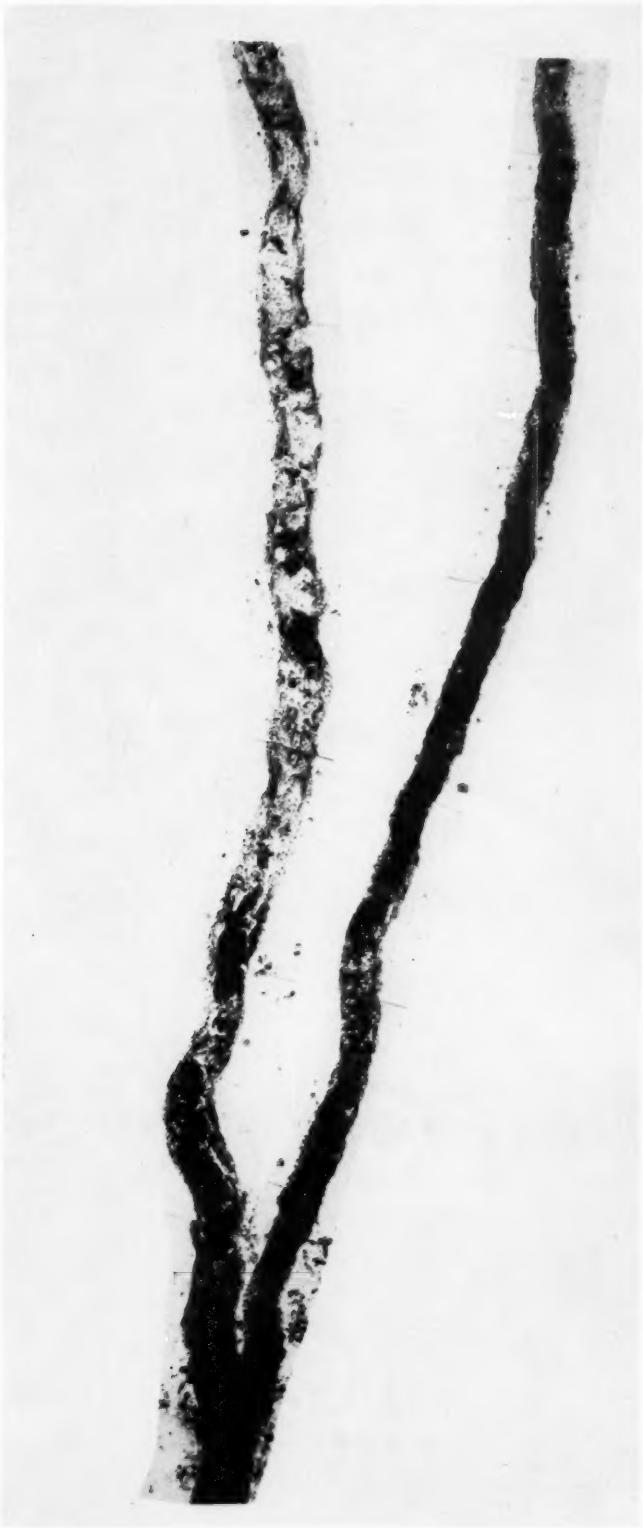


PLATE X (A TO B). PORTION OF PROXIMAL CONVOLUTION FROM CASE 33 WHO DIED ON THE NINETEENTH DAY OF HIS ILLNESS AND IN THE TENTH DAY OF DIURESIS

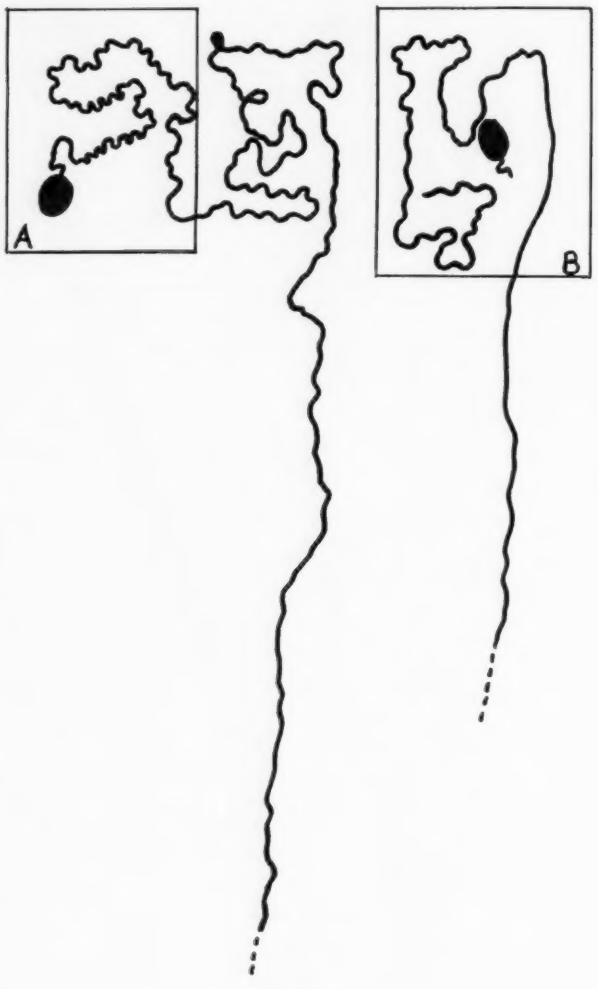


PLATE XA

Although the lumens of the proximal convolutions appear large in histological section (*cf.* Figure 40), as can be seen from the dissected specimen, this is not due to dilatation of the tubule but to the irregular regeneration of its epithelial wall (*a*). Original magnification of 200 × is here reduced to 80 ×.

a

PLATE XB. THE DISTAL CONVOLUTION OF THE SAME NEPHRON

A duplicate print of the glomerulus has been mounted in the position of its attachment to the tubule. The ascending limb is essentially normal. The first half of the distal convolution is irregularly dilated, the second half filled with a large solid cast-like mass which continues into the connecting tubule.

PLATE XI (A TO B). THE TERMINAL COLLECTING TUBULE AND THE DUCTS OF BELLINI FROM THE SAME CASE

In the normal kidney these tubules show smooth, even contours with gradually increasing diameters. These are markedly irregular both from the presence of the large renal failure casts of Addis that intermittently distend their lumen and from the irregular hyperplastic proliferation of their epithelial cells. Original magnification of 100 × is here reduced to 40 ×.

PLATE XIB. THE DUCTS OF BELLINI ENTERING THE RENAL PELVIS

The foot-like appendage that joins the three ducts below is a reflection of the pelvic epithelium which usually remains attached in spite of dissection. As has been observed, the tortuosity and irregularity of contour is seen to be due to the masses of debris that fill the lumens as well as to the marked hyperplasia of epithelial cells of the walls of ducts. For histological appearance, *cf.* Figure 41. Original magnification of 175 × is here reduced to 80 ×.